THE STRUCTURE AND CHEMISTRY OF ALGINIC ACID.

By

John W. B. Samuel B.Sc.

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Part of this work has been published. A reprint is inserted at the end of this Thesis.

CONTENTS.

GENERAL INTRODUCTION.

Alginic acid is one of the main carbohydrates of the Phaeophycae, the brown seaweeds, its function not being clear. The proportion varies with the season, species of Laminaria containing 24% of alginic acid in February and only 14% in September (1). To isolate it, the cleaned weed is first steeped in dilute acid, washed and then extracted with sodium carbonate solution, when a solution of sodium alginate is obtained. This material finds many commercial uses (2). It finds uses in cold setting jellies, as a stabiliser in many foods, particularly ice cream, as a thickener in textile printing, in the surface sizing of paper and in water purification.

Alginic acid is a high molecular weight polysaccharide and until 1955 it was thought that it contained only residues of D-mannuronic acid. However, Fischer and Dorfel (3) showed that hydrolysis gave L-guluronic acid, the C-5 epimer of D-mannuronic, as well as D-mannuronic acid. Methylation analysis indicates that both these units are 1,4-linked (4), a conclusion that is substantiated by the application of other methods of structural investigation (5,6), and with which results of earlier work are consistent (7,8).

Isolation of 4-O-β-D-mannopyranosyl-D-mannopyranose (9) from a partial hydrolysate of the reduced polysaccharide indicates that the mannuronic acid residues in alginic acid are linked through their C-4 positions by a β-linkage and that the linkage is 1,4-pyranosyl rather than 1,5-furanosyl. Partial fractionation into fractions rich in mannuronic and guluronic acids, respectively, has been achieved, (10,11) but repeated fractionation failed to separate a polymer which contained only mannuronic or guluronic acid residues. Proof that the two acids appear together in at least some of the alginic acid molecules was

supplied by the isolation of oligouronic acids (12) containing both acids, and of a crystalline mannosyl-gulose (9) from partial acid hydrolysates of alginic acid and its reduction product respectively.

A study of the constitution of alginic acid by partial acid hydrolysis has been carried out by Haug, Smidsrød and Larsen (13). Heterogeneous hydrolysis of the alginate was carried out with oxalic acid. Results showed that a certain amount of the alginate passed rapidly into solution, but even prolonged hydrolysis did not increase the concentration of carbohydrate in the solution to more than corresponding to 28% of the alginate. This clearly indicated that only part of the alginate sample was available for hydrolysis, while the rest of the sample was protected against hydrolysis or hydrolysed very slowly. The insoluble material could only be further degraded when it was washed, dissolved in dilute alkali and then retreated with oxalic acid. Even then there was a limit to the amount of hydrolysis taking place. The insoluble fraction could be fractionated into one fraction which contained predominantly guluronic acid residues and another which contained predominantly mannuronic acid residues. Significantly, no fraction with an intermediate uronic acid composition could be prepared. The number average length of the insoluble chains was 20-30. The soluble fraction was shown tentatively to consist predominantly of the two monomers and a diuronide containing both the monomers. From these results Haug, Smidsrød and Larsen deduced that alginic acid consists of blocks of 20-30 monomer units with either predominantly mannuronic or guluronic acids and that these blocks are separated by regions with another sequence of uronic acid residues, probably with a large proportion of alternating mannuronic and guluronic acid residues. The blocks with a highly regular structure more easily form crystalline regions with a

much lower rate of hydrolysis than the more amorphous regions.

It has still to be shown conclusively whether structural irregularities, such as branching or non-1,4-linking, ever occurs in the molecule. The incomplete oxidation of sodium alginate by periodate (6) would be explained if such irregularities were present. These questions are more fully examined in Section A. Another unsolved problem is the configuration of the guluronosyl linkage.

It is noteworthy that a bacterial polysaccharide resembling alginic acid has been isolated from Azotobacter vinelandii (14) and Pseudomonas aeruginosa (15,16). Studies are not yet so complete as on algal alginic acid and there would seem to be a close structural similarity except that the bacterial polymer is at least sometimes O-acetylated.

Degradation of alginate by a β-elimination reaction has been achieved both enzymically (17,18) and chemically (19) (see Section C), and oxidative degradation by a free-radical mechanism by naturally-occuring phenolic compounds has also been shown to take place (20,21).

In some ways, alginic acid has a more simple structure than pectic acid, a related uronic acid polymer from higher plants, in which the monomer is galacturonic acid. Pectin (22) is similar to alginic acid in that it contains a backbone of uronic acid residues, but blocks of polygalacturonic acid appear to be interrupted by occasional neutral residues (rhamnose). Neutral side chains are also present in varying degree, depending on the source of the pectin. Unlike pectic acid, alginic acid does not occur in the esterified state, nor do neutral sugars ever seem to be part of the molecule. The chemical reactions of alginic acid are therefore of interest, not only for their own sake, but also because they might usefully be applied in the structure

determination of the more complex pectic substances. One of the aims of the work which is reported in this Thesis, has been to use alginic acid to develop new approaches to the structure determination of uronic acid-containing polysaccharides. Details of these approaches are given in Sections B and C.

X-ray analysis of alginic acid gives well-developed diffraction patterns (23,24), but it has since been shown by Frei and Preston (25) that the sample examined was in fact a guluronic acid-rich sample. The data obtained therefore corresponded to polyguluronic acid and not to polymannuronic acid as was originally supposed. If we ignore the C-6 carbon atom then polyguluronic acid has the same carbon skeleton as cellulose (as well as xylan, mannan and polymannuronic acid) and these two polymers are indeed related in having a 2-fold screw axis along the fibre axis although the fibre repeat distance is somewhat shorter for polyguluronic acid - 8.7Å as opposed to 10.3Å for cellulose. Frei and Preston reached the conclusion that the algal cell wall contains mainly material rich in guluronic acid whereas the intercellular alginic acid is primarily polymannuronic acid.

The physical properties of alginate solutions are in many ways similar to those of pectins in the higher plant kingdom and to those of the sulphated polysaccharides from the red seaweeds e.g. K-carrageenan. For example, all form strong, reversible, cation-sensitive gels. This similarity, and the fact that all these polysaccharides occur at least partly in the intercellular parts of plant tissue, suggest that they might have similar biological functions. Accordingly, in seeking to understand the conformation, and the physical and biological properties of alginic acids, it would seem worthwhile to study all three types of polysaccharide together. Some progress has been made in this laboratory

towards the determination of the conformation of K-carrageenan (26) by X-ray diffraction; this is described later in this Thesis (Section D) together with the results of an attempt to develop the X-ray methods further and to apply them to alginic and pectic acids.

GENERAL METHODS OF INVESTIGATION.

Concentration of Polysaccharide Solutions. Solutions were concentrated under reduced pressure using a rotary film evaporator with a bath temperature of 40° or less.

Dialysis. Polysaccharide solutions were dialysed in cellophane tubes suspended in running tap water. Chloroform was added to prevent bacterial action.

Electrophoresis. This was carried out in pyridine-acetic acid buffer (0.1M) at pH 6, with a voltage gradient of 14 volts/cm. Picric acid was used as a marker.

Hydrolysis of Polysaccharides. Unless otherwise stated, the material (about 5mg) was sealed in a tube with aqueous formic acid (45%; about 1ml) and heated at 100° for about 16 hours. After evaporation of the solution to dryness, any formyl esters formed were removed by addition of water (about 2ml) and re-evaporation to dryness (3 times).

Methanolysis. Samples of methylated sugars (about 2mg) were heated in sealed pyrex test tubes at 100° for 6 hours with 3% methanolic hydrogen chloride. The solution of methyl glycosides was neutralised with silver carbonate, filtered and evaporated to dryness.

Borohydride Reduction. Sugars (about 5mg) were dissolved in water and left overnight at room temperature with potassium borohydride. Excess borohydride was destroyed with IR-120 (H+) resin. The solution was then filtered, evaporated to dryness and boric acid was removed as methyl borate by repeated distillation with methanol.

Preparation of Acetylated Monosaccharides for Gas-Liquid Chromatography. Sugars (about 5mg) were treated with acetic anhydride (0.2ml) and pyridine (0.4ml) for 1 hour at 100°. After evaporation to dryness, residual pyridine was removed by repeated distillation with methanol.

The product was dissolved in chloroform for examination by gas-liquid chromatography.

Preparation of Glycol Esters. A solution of the polysaccharide in the free acid form was treated with the appropriate oxide until the solution was neutral - (length of treatment required was variable, but was generally in the region of 8 weeks). After dialysis, the solution was concentrated and freeze-dried.

Methoxyl Contents. These were determined by A. H. Baird using the Zeisel method (27).

Ash Contents. These were estimated by ignition of samples (about 50mg) to constant weight in a silica crucible.

Paper Chromatography. Whatman No.1 or No.4 paper was used and the following solvent systems were used:-

1. ethyl acetate : pyridine : water	10 : 4 : 3
2. n-butanol : ethanol : water (upper layer)	4 : 1 : 5
3. ethyl acetate : pyridine : acetic acid : water	5 : 5 : 1 : 3
4. n-butanol : ethanol : water	1 : 1 : 1
5. ethyl acetate : acetic acid : formic acid : water	18 : 3 : 1 : 4
6. methyl ethyl ketone : water : formic acid	200 : 17 : 1
7. n-butanol : acetic acid : water	50 : 12 : 25
8. methyl ethyl ketone : acetic acid : water (saturated with boric acid).	9 : 1 : 1

The following detection sprays were used:-

1. p-anisidine hydrochloride (28) (for reducing sugars). The dried chromatograms were sprayed with a 3% solution of p-anisidine hydrochloride in water-saturated n-butanol, and heated at about 120° for 5 minutes.

2. periodate-benzidine (29) (for compoumds containing glycol groups). A 2.28% aqueous solution of periodic acid (1ml) was diluted with

acetone (29ml). The chromatogram was passed through this solution, left at room temperature for 7 minutes, and passed through a solution prepared by dissolving benzidine (1.84g) in a mixture of acetic acid (6ml) and acetone (1 litre). White spots were obtained on a blue background after a few minutes at room temperature.

3. periodate-Schiff (30) (for compounds containing glycol groups). The dried chromatogram was sprayed with an aqueous solution of sodium periodate (0.3%, w/v) and left at room temperature for 10 minutes; unused periodate was destroyed with sulphur dioxide gas, and then the chromatogram was sprayed with Schiff's reagent (made by dissolving p-rosaniline hydrochloride (1g) in water (100ml) which had been saturated with sulphur dioxide).

4. thiobarbituric acid (31) (for 4,5-unsaturated uronic acids). The dried chromatogram was sprayed with 0.02M sodium periodate. After 15 minutes the paper was sprayed with a mixture of ethylene glycol : acetone : concentrated sulphuric acid (50 : 50 : 0.3) and after a further 10 minutes with aqueous 6% sodium thiobarbiturate. Red spots appeared after heating at 100° for 5 minutes and were especially sensitive under ultra-violet light.

5. aniline-xylose (32, page231) (for uronic acids). Xylose (1g) was dissolved in the minimum volume of water, then aniline (1ml) was added. The solution was then dissolved in ethanol (about 100ml) and the chromatogram was sprayed evenly then heated at 110° for 5 minutes.

Gas- liquid Chromatography. This was carried out with a Pye Argon chromatograph fitted with a strontium-90 detector. 0.1µl samples of the materials in chloroform solution were examined. The column packings used are described in the experimental section.

Thin Layer Chromatography. This was carried out on a small scale by

using a microscope slide as the plate and a small bottle as the solvent tank. The coating used was silica gel (Kieselgel G. nach Stahl, Merck). The solvent used was methanol : benzene (in varying proportions) and the spots were developed by spraying with a solution made up of anisaldehyde (1ml), sulphuric acid (AnalaR, 1ml), and ethanol (30ml) followed by heating at 150° for a few minutes.

Determination of Carbohydrate Content (33). The carbohydrate solution (1ml, containing about 100µg carbohydrate) was placed in a test tube and aqueous phenol (5% w/v, 2ml) was added. Concentrated AnalaR sulphuric acid (5ml) was then added from a fast delivery pipette. When cool, the optical density of the solution was measured with an EEL colorimeter using filter no. 623 (maximum transmission at 495mµ), and the amount of sugar present calculated by reference to a calibration curve previously prepared using a standard solution of the appropriate sugars.

SECTION A.

METHYLATION SURVEY OF ALGINIC ACID.

INTRODUCTION.

Methylation analysis is potentially the most useful method for obtaining information about the structural units present in polysaccharides, but only recently (4) has it been possible to identify unequivocally any methylated sugar after hydrolysis of methylated alginic acid. Early methylation studies showed the presence of 1,4-linked uronic acid residues in the polymer but since mannuronic acid was the only unit known to be present at that time the conclusions which were drawn from these results are different from those which would have been drawn had the presence of guluronic acid been known.

The isolation of di-O-methylerythraric acid (7) from degradation of the methylated sugar(s) was taken to show that 2,3-di-O-methyl-D-mannuronic acid units had been present in the methylated polysaccharide. However, di-O-methylerythraric acid could have been derived from the 2,3 di-O-methyl ether of either mannuronic acid or guluronic acid or from a mixture of both. The failure to prepare a crystalline derivative of 2,3-di-O-methyl-D-mannose (8) and the isolation of a di-O-methyl-D-glucose also remained unexplained. More recent work (4) has shown that 2,3-di-O-methyl-D-mannose and 2,3-di-O-methyl-L-gulose are inseparable in the solvent systems used for chromatography by the earlier workers, as are the parent sugars, mannose and gulose, which would be obtained from them by demethylation. This would explain how the gulose derivative escaped the notice of the earlier workers and also why the 2,3-di-O-methyl-D-mannose fraction (presumably containing 2,3-di-O-methyl-L-gulose) isolated by them after column chromatography did not form a crystalline derivative despite repeated attempts (34). A further

complication is the fact that gulose forms a 1,6-anhydride and this would account for the 'methyl 2,3-di-O-methylmannoside' which was isolated after cellulose column chromatography and which resisted complete acid hydrolysis. This presumably was 1,6-anhydro-2,3-di-O-methyl-β-L-gulopyranose which cannot be completely hydrolysed by acid to the free sugar but only equilibrated with it..

Unambiguous characterisation of 2,3-di-O-methyl-D-mannose and 1,6-anhydro-2,3-di-O-methyl-β-L-gulopyranose (4) after hydrolysis and reduction of methylated alginic acid firmly indicated that they arose from β-1,4-linked mannuronic acid and 1,4-linked (possibly α) guluronic acid and that these units were the major structural units in the polymer. It was also reported that it was highly unlikely that any branching occured in the molecule since mono- and tri-methyl ethers, which would have arisen as a result, were not present on paper chromatography. However the presence of small amounts of non-1,4-linked units could not be ruled out on the basis of the results obtained. It should also be noted at this point that methylation analysis does not distinguish between 1,4-pyranosyl and 1,5-furanosyl linkages although the presence of the latter is unlikely because of the high resistance to acid hydrolysis of the polysaccharide.

The basic aim of the work described in this section was to determine, by methylation analysis, the structural variation in alginate samples from varying sources and having varying mannuronic acid/guluronic acid ratios.

It is well-known that the mannuronic acid/guluronic acid ratio affects the relative binding power of alginic acid to calcium and sodium (35) so that quantitative differences are found in the rheological behaviour of alginates from different sources in those

regions where the proportions of calcium and sodium are important. However the question arises whether the differences in physical properties are due solely to different mannuronic acid/guluronic acid ratios. In fact, Haug (36) has found an alginate sample from Ascophyllum nodosum with the same mannuronic acid/guluronic acid ratio as alginate from Laminaria digitata and having different physical properties. The possibility that structural differences, such as branching and/or non-1,4-linking, might be causing the differences in physical properties was therefore investigated.

Another sample which was thought to be worthwhile investigating was a sample which had been fully periodate oxidised prior to methylation. It has been shown by Drummond, Hirst and Percival (6), that a high proportion of both mannuronic acid and guluronic acid units in the polymer are periodate resistant. They suggested that since all the evidence pointed to a 1,4-linked chain then the immunity might be due to residues in the polymer of the 3,6 lactone of mannuronic acid. Another possibility they suggested was that cross-linking between the carboxyl group of one chain and the C-2 or C-3 hydroxyl group of an adjacent chain to form an ester linkage, would render a proportion of the residues immune to oxidation. They however did not discard the possibility of 1,3 linkages. By examination of this sample it was hoped to be able to show if the resistant units do in fact arise from any of the above possibilities.

By obtaining methods for the separation of all the possible 2,3-di-O-methyl and 2,3,4-tri-O-methyl ethers which can arise after reduction of the hydrolysed methylated polysaccharide it was hoped to be able to show if the proportion of tri-O-methyl sugars, i.e. end-group units, was enough to indicate any branching in the polysaccharide.

It was also hoped to be able to differentiate between the three di-O-methyl sugars which can arise from linking at C-2, C-3, or C-4, so that any non-1,4 links might be detected.

A major difficulty was the fact that the only methylated derivative of gulose which has been reported is 1,6-anhydro-2,3-di-O-methyl-β-L-gulopyranose (4). An authentic sample of the 4-O-p-nitrobenzoyl ester of this compound made a convenient starting compound for the synthesis of the 2,3-di-O-methylgulose and 2,3,4-tri-O-methylgulose derivatives required in the first of the above experiments. However the detection of other methylated gulose derivatives in the second of the above experiments was obviously out of the question so that a straightforward methylation analysis could not be carried out due to a lack of standard compounds. Another series of reactions had therefore to be devised to sort out this problem.

After this work was complete an investigation was reported which used similar methods in the investigation of bacterial alginic acid. This polysaccharide was an exocellular polysaccharide from Azotobacter vinelandii and consisted of mainly D-mannuronic acid, with a small proportion of L-guluronic acid units. This polysaccharide differed from algal alginic acid in that it was partly acetylated.

The deacetylated polysaccharide showed a striking resemblance to alginic acid in that it was only partially oxidised with sodium periodate. After reduction of the polyaldehyde with sodium borohydride the derived polyalcohol was fully methylated. Hydrolysis and reduction gave a di-O-methyl mannose which was shown to be 2,3-di-O-methylmannose by isolation, after periodate oxidation, of 2,3-di-O-methylerythrose. No other products were obtained. These results indicated that the bacterial polyuronide polyaldehyde contained only 4-O-pyranosyl

and/or 5-O-furanosyl mannuronic acid units and it would seem that the immunity is due to some form of tight association between chains in the molecule.

EXPERIMENTAL.

Experiment 1. Development of methods for the separation of 2,3-di-O-methylmannose and 2,3-di-O-methylgulose.

1(a). Preparation of 2,3-di-O-methylgulose.

1,6-anhydro-2,3-di-O-methylgulose (100mg) was dissolved in a mixture of acetic anhydride (5ml) and concentrated sulphuric acid (0.05ml) and left to stand at room temperature for 20 hours. The solution was poured into water (100ml) and treated with solid sodium bicarbonate until it was neutral and effervescence ceased and then extracted with chloroform. The chloroform was dried (sodium sulphate) and evaporated to a syrup which was dissolved in dry methanol (6ml) containing sodium (2mg). Thin layer chromatography showed that the deacylation reaction was complete in 10 minutes and that the sole product had the mobility of di-O-methyl gulose with no anhydride detectable. Excess of solid carbon dioxide was added and the solution was concentrated to dryness.

1(b). Paper chromatography of the di-O-methyl sugars.

Hirst and Rees (4) had previously shown that there was no separation in several solvent systems and the following solvent systems also proved unsuccessful.

methyl ethyl ketone : water : concentrated ammonia	200 : 17 : 1
benzene : ethanol : water	169 : 47 : 15

1(c). Thin layer chromatography of the di-O-methyl sugars.

This technique was tried after methanolysis of the two di-O-methyl sugars. The methyl glycosides themselves did not separate although the presence of the gulose derivative could be shown since its 1,6-anhydride moved appreciably faster.

1(d). Gas-liquid chromatography of the di-O-methyl sugars.

The two di-O-methyl sugars were completely resolved as the fully

acetylated glycitols, on a 3% XE-60 column (on Gas-chrom P) at 200°. The methyl glycosides prepared from the di-O-methyl sugars by methanolysis were not resolved on a polyphenyl ether column, and were retained by the neopentyl glycol adipate (NPGA) (3% on Gas-chrom P) column, even at 225°. Acetylation of the glycosides gave products that were well separated on the XE-60 column at 175°, but only partly separated on the NPGA column. In both the successful methods of separation (see General Methods), the derivative of 1,6-anhydro-2,3-di-O-methylgulose was also fully resolved as were the corresponding derivatives of 2,3,4-tri-O-methylmannose.

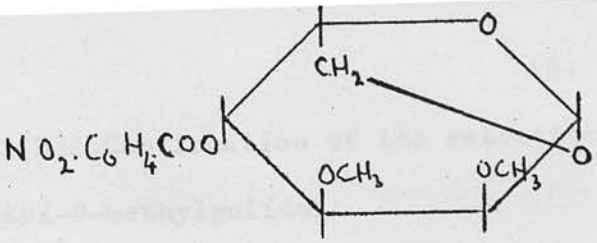
It should be noted that the use of analytical grade ion-exchange resin (IR-120) was found to be essential in the preparation of derivatives for gas-liquid chromatography. Ordinary grade resin gave rise to substantial amounts of extraneous peaks on gas-liquid chromatograms.

Experiment 2. Preparation and gas-liquid chromatography of 2,3,4-tri-O-methylgulose derivatives.

2(a). Preparation and gas-liquid chromatography of 2,3,4-tri-O-methyl-gulose derivatives.

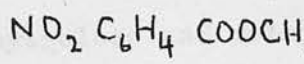
1,6-anhydro-2,3-di-O-methyl-4-O-p-nitrobenzoyl-L-gulose (20mg) was de-esterified by shaking at room temperature overnight with sodium methoxide in methanol (0.1%; 5ml). After neutralisation (solid carbon dioxide) thin layer chromatography showed that de-esterification had gone to completion. The solution was evaporated to dryness and to the residue was added dimethylsulphoxide (0.5ml) and dimethylformamide (0.5ml) (37). The mixture was shaken in ice (15 minutes) before addition of barium hydroxide octahydrate (0.5g) and then dimethyl sulphate (0.35ml). After shaking in ice for a further 2 hours, the mixture was

removed and shaken at room temperature for 2 days. Concentrated ammonia (0.2ml) was added and shaking was continued for 30 minutes. The product was isolated by dilution with water (3 or 4 volumes), followed by extraction into chloroform. The chloroform layer was washed until the washings were neutral, then dried over sodium sulphate. Gas-liquid chromatography (XE-60 column at 175°) showed two peaks, one of which (the slower) corresponded to methyl nitrobenzoate when compared with a mixture previously prepared (38) and which contained this as one component. The faster peak was presumably due to 1,6-anhydro-2,3,4-tri-O-methylgulose. After methanolysis and acetylation this mixture was re-examined by gas-liquid chromatography to give the retention time of the acetylated methyl glycoside of 2,3,4-tri-O-methylgulose. Part of the methanolysate was hydrolysed (45% formic acid at 100° for 8 hours followed by evaporation to dryness; The residue was dissolved in 1N sulphuric acid and heated at 100° overnight, then the solution was neutralised with calcium carbonate, filtered and deionised with a mixture of IR-120 and IR-45 resins) and analysed by gas-liquid chromatography after reduction (potassium borohydride) and acetylation. The product was a mixture of 1,6-anhydro-2,3,4-tri-O-methylgulose and 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylgulitol. A peak was present which had a similar retention time to 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylmannitol and this was assigned to the tri-methylgulitol derivative. The methyl nitrobenzoate peak was absent and there were two new peaks with retention times which were of the same order but distinctly different. It was thought that these were probably derived from methyl nitrobenzoate but further investigation was considered necessary. The sequence of the above reactions is shown in the accompanying diagrams (figure 1).



1,6-anhydro-2,3-di-0-methyl-4-0-p-nitrobenzoyl-L-gulose

sodium in methanol



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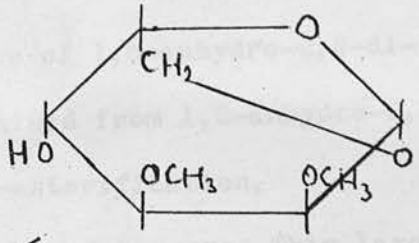
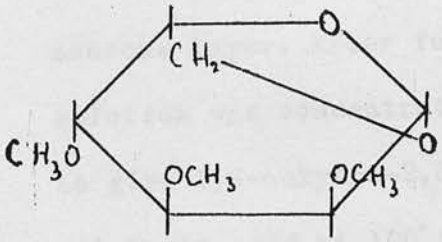


methyl p-nitrobenzoate

1,6-anhydro-2,3-di-0-methylgulose

dimethylsulphate

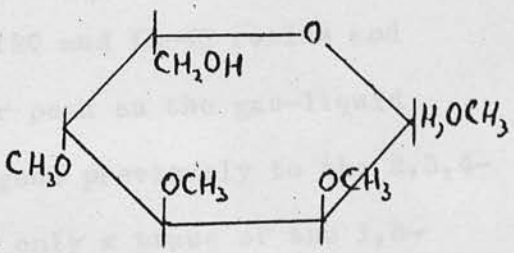


1,6-anhydro-2,3,4-tri-0-methylgulose

methanolysis

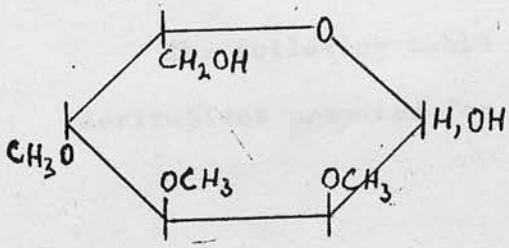
1,6-anhydro-2,3,4-tri-0-methylgulose

+



methyl 2,3,4-tri-0-methyl-guloside

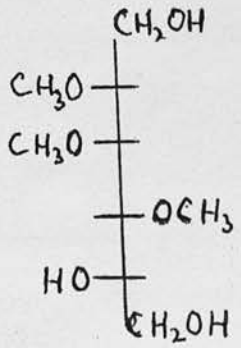
hydrolysis



2,3,4-tri-0-methylgulose

+ 1,6-anhydro-2,3,4-tri-0-methylgulose

reduction



2,3,4-tri-0-methylgulitol

Figure 1.

2(b). Confirmation of the retention time of 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylgulitol.

Since the above retention time was based on the assignment of one particular peak in a mixture of substances the following experiment was aimed at confirming the assignment. The mixture of 1,6-anhydro-2,3-di-O-methylgulose and methyl p-nitrobenzoate obtained from 1,6-anhydro-2,3-di-O-methyl-4-O-p-nitrobenzoyl-L-gulose by de-esterification, (experiment 2(a)) was partitioned between water and benzene. Thin layer chromatography showed the absence of methyl p-nitrobenzoate from the aqueous layer. After further extraction with benzene, the aqueous solution was concentrated to a syrup which was then methylated as before to give 1,6-anhydro-2,3,4-tri-O-methylgulose. After hydrolysis with 1N sulphuric acid at 100° for 16 hours to establish equilibrium between the sugar and the anhydride, the solution was neutralised with calcium carbonate, deionised with a mixture of IR-120 and IR-45 resins and reduced and acetylated as before. The major peak on the gas-liquid chromatogram corresponded to the peak assigned previously to the 2,3,4-tri-O-methylgulitol derivatives; there was only a trace of the 1,6-anhydride.

The following table gives the relative retention times of the derivatives prepared for the survey.

Table 1.

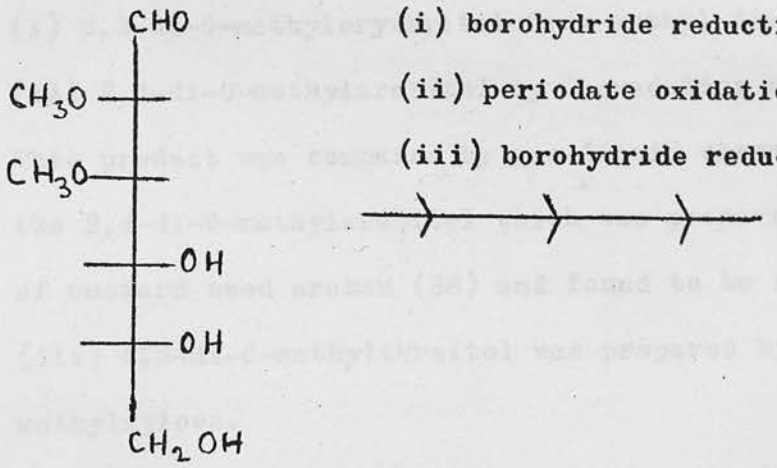
Parent methylated sugar.	Relative retention time of product of methanolysis and acetylation (column at 175°)	Relative retention time of product of reduction and acetylation (column at 200°)
1,6-anhydro-2,3,4-tri-0-methylgulose	0.17	0.08
2,3,4-tri-0-methyl-gulose	0.42	0.62
2,3,4-tri-0-methyl-mannose	0.36	0.61
1,6-anhydro-2,3-di-0-methylgulose	0.50	0.23
2,3-di-0-methylgulose	0.61	1.10
2,3-di-0-methylmannose	1.00	1.00
Extraneous peak*	-	0.39

*Note: This extraneous peak appeared in some of the samples and also (in varying degree) in some of the standards.

Experiment 3. Development of a method for the detection of isomeric di-0-methyl mannoses and di-0-methyl guloses.

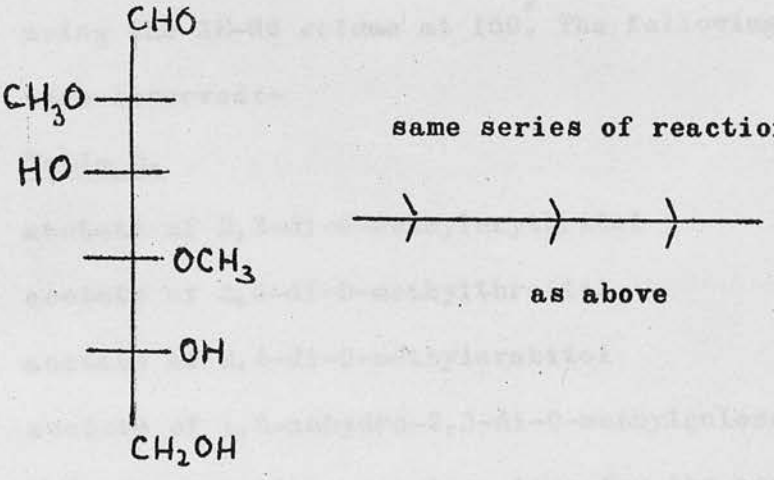
The series of reactions shown in figure 2 was devised to confirm the non-existence of the 2,4- and 3,4-di-0-methyl derivatives of mannose and gulose after reduction of hydrolysates of methylated alginic acids.

Exactly the same products would be obtained from the corresponding gulose derivatives, because the only difference between mannose and gulose is the configuration at C-5 and the dissymetry at this centre is destroyed in the reactions.



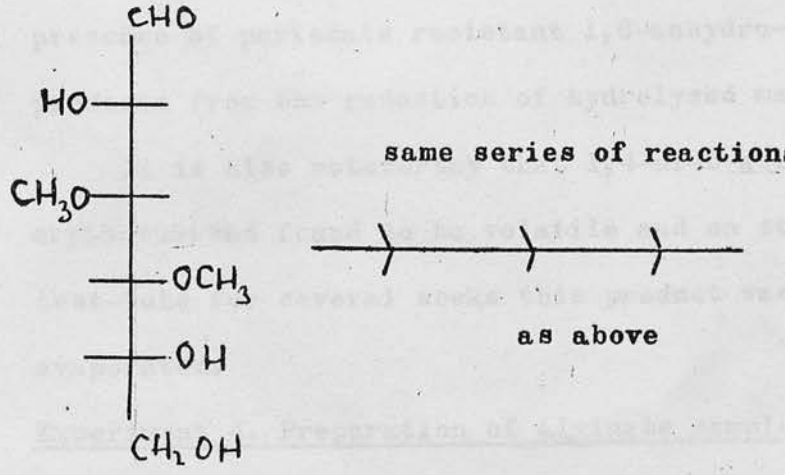
2,3-di-O-methylmannose

2,3-di-O-methylerythritol



2,4-di-O-methylmannose

2,4-di-O-methylarabitol



3,4-di-O-methylmannose

2,3-di-O-methylthreitol

Figure 2.

The expected products were prepared as shown in figure 3.

(i) 2,3-di-O-methylerythritol from methyl di-O-methylerythrarate

(ii) 2,4-di-O-methylarabitol by degradation of 2,4-di-O-methylgalactose. This product was compared by gas-liquid chromatography (see below) with the 2,4-di-O-methylarabitol which was prepared in connection with studies of mustard seed araban (38) and found to be indistinguishable from it.

(iii) 2,3-di-O-methylthreitol was prepared by degradation of 2,3-di-O-methylxylose.

Each of the products was acetylated with acetic anhydride and pyridine at 100° (1 hour) and then examined by gas-liquid chromatography, using the XE-60 column at 150°. The following relative retention times were observed:-

Table 2.

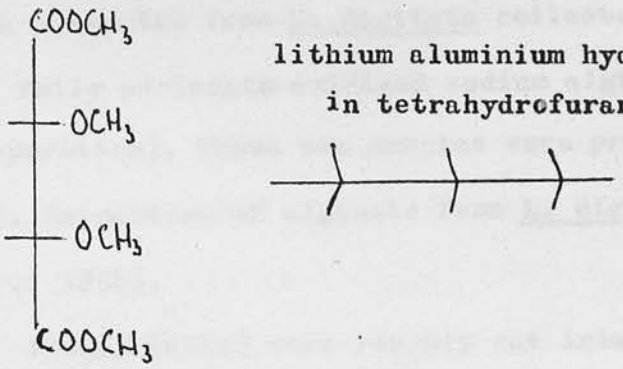
acetate of 2,3-di-O-methylerythritol	0.15
acetate of 2,3-di-O-methylthreitol	0.18
acetate of 2,4-di-O-methylarabitol	1.00
acetate of 1,6-anhydro-2,3-di-O-methylgulose	0.47

N.B. The relative retention time for the acetate of 1,6-anhydro-2,3-di-O-methylgulose is given since this product is expected due to the presence of periodate resistant 1,6-anhydro-2,3-di-O-methylgulose in the products from the reduction of hydrolysed methylated alginic acid.

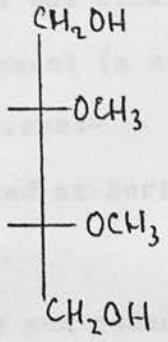
It is also noteworthy that 1,4-di-O-acetyl-2,3-di-O-methyl-erythritol was found to be volatile and on standing in a lightly stoppered test-tube for several weeks this product was found to be almost entirely evaporated.

Experiment 4. Preparation of alginate samples.

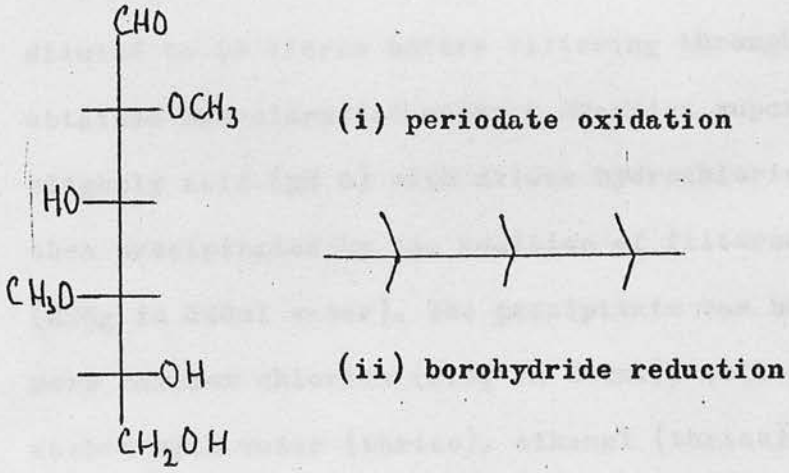
Eight of the ten samples examined were available as either the free acid or as the sodium salt and were therefore methylated after



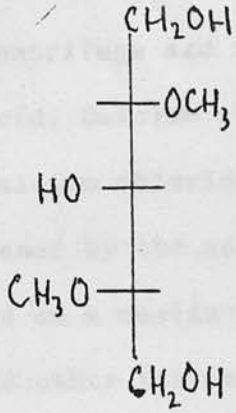
methyl di-O-methylerythrarate



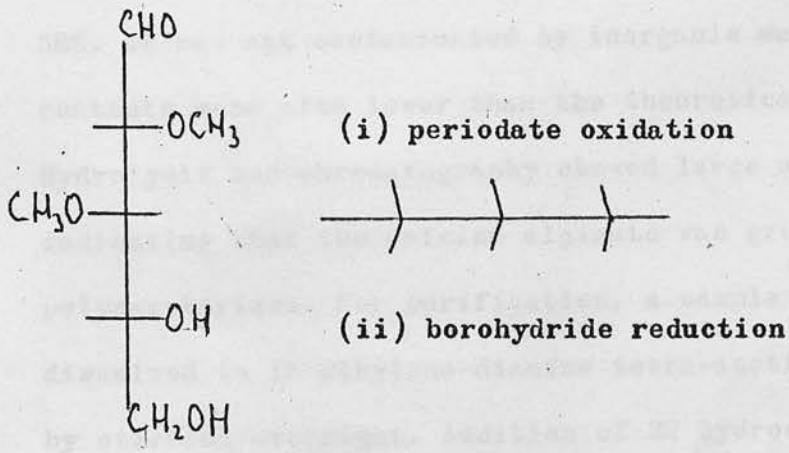
2,3-di-O-methylerythritol



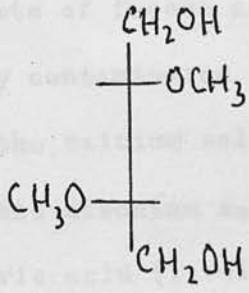
2,4-di-O-methylgalactose



2,4-di-O-methylarabitol



2,3-di-O-methylxylose



2,3-di-O-methylthreitol

Figure 3.

dissolution in potassium hydroxide. One sample was an alginate which had been extracted from L. digitata collected locally and the final sample was fully periodate-oxidised sodium alginate from Manucol (a commericial preparation). These two samples were prepared as follows:-

4(a). Extraction of alginate from L. digitata collected at North Berwick (April 1965).

Fronds (8lbs) were roughly cut into small pieces and added to aqueous sodium carbonate (150g in 12l). After stirring at room temperature overnight, a thick viscous mass was obtained and this was diluted to 26 litres before filtering through muslin. The liquor obtained was clarified using a Sharples super-centrifuge and then made slightly acid (pH 6) with dilute hydrochloric acid. Calcium alginate was then precipitated by the addition of filtered calcium chloride solution (225g in 520ml water). The precipitate was hardened by the addition of more calcium chloride (110g in 250ml), collected on a muslin filter and washed with water (thrice), ethanol (thrice) and ether (thrice). Finally it was dried on filter sheets and then in a dessicator. (Yield, 30g). The product had a uronic anhydride content (by decarboxylation) of only 58%. It was not contaminated by inorganic material, because the ash contents were also lower than the theoretical for calcium alginate. Hydrolysis and chromatography showed large amoumts of fucose and xylose, indicating that the calcium alginate was grossly contaminated by other polysaccharides. For purification, a sample of the calcium salt was dissolved in 1% ethylene-diamine tetra-acetic acid disodium salt (EDTA) by stirring overnight. Addition of 2N hydrochloric acid (1 volume) gave a precipitate of alginic acid that was washed six times with 1N hydrochloric acid and then with distilled water until the washings were neutral. Hydrolysis and paper chromatography showed that the product

was therefore suitable for methylation.

4(b). Periodate oxidation of the sodium salt of Manucol. (carried out by Dr. D. A. Rees).

Sodium alginate (9.0g) was dissolved in water and sodium periodate (32.1g = 3 moles/hexuronic acid unit) was added. After dilution with water to 1 litre, the solution was left at room temperature and the reduction of periodate was followed spectrophotometrically (39). The following values were obtained:

Time (hours)	2	3	10	60
Periodate reduced (mol)	0.44	0.51	0.51	1.62

Ethylene glycol (2ml) was added after 60 hours, and then potassium borohydride (10g) was added after 16 hours, and 4 hours later the solution was chilled to 0° and dimethyl sulphate and sodium hydroxide (30% w/v; 150ml) were added under nitrogen with vigorous stirring in the usual way. Further additions of dimethyl sulphate and sodium hydroxide were made until methylation was complete, the solution being dialysed and concentrated under reduced pressure when necessary. (Yield 3.5g). The criterion of complete methylation was that reduction and hydrolysis (see experiment 5(a)) showed negligible quantities of non-methylated and monomethylated sugars on paper chromatography.

Experiment 5. Survey of the structural units present in different samples of alginates.

5(a). Methylation of the alginate samples and conversion to the corresponding mixture of methylated neutral sugars.

Samples of the various alginates (1-2g) were converted to their fully methylated derivatives by repeated treatment with dimethyl sulphate and potassium hydroxide until the theoretical degree of etherification had been achieved. Fully methylated alginate samples

(50mg of the potassium salt) were then partially hydrolysed with 50% formic acid (2ml) for 8 hours at 100°. After evaporation to dryness and dissolution in water, cations were removed with IR-120. After re-evaporation to dryness the residues were dried thoroughly in a vacuum oven at 60° then 3% methanolic hydrogen chloride (1-2ml) was added and boiled under reflux for 6 hours. The solutions were neutralised (silver carbonate), filtered, and concentrated to dryness. Reduction was effected by treatment with lithium aluminium hydride (20-30mg) in tetrahydrofuran (2-3ml) for 16 hours at room temperature then 4 hours under reflux. Excess of hydride was destroyed by the addition of ethyl acetate and the minimum quantity of water was added to precipitate lithium aluminate. The solutions were filtered and evaporated to dryness, and the residue heated with 1N sulphuric acid (2ml) to achieve complete hydrolysis. After neutralisation (calcium carbonate) and deionisation (IR-120 and IR-45 resins) the solutions were evaporated to give golden brown syrups.

A list of the samples examined is given in Table 3, together with details about them and the results of methoxyl analysis of their fully methylated derivatives.

Table 3.

Sample Number	Source	Mannuronic/ Guluronic Ratio	% - OCH_3
1	L. digitata, new fronds, Reine 10/2/64.	2.2	25.1
2	L. digitata, old fronds, Reine 10/2/64.	1.35	24.8
3	L. hyperborea stipes, Hustad 4/5/64.	0.5	27.4
4	Ascophyllum nodosum, Vaere 2/3/64.	1.6	26.4
5	Commercial preparation (Manucol)		26.1
6	L digitata (ex Marine Colloids), March 1966.		25.2
7	L.digitata, North Berwick, April 1965.		26.3
8	Periodate-oxidised Manucol		
9	L. digitata ex Chanda et al 1952.		25.8
10	L. cloustonii ex Hirst and Rees 1965.		25.0

N.B. The theoretical methoxyl content of fully methylated potassium alginate is 25.6%.

Samples 9 and 10 were samples which were available from the work of Chanda, Hirst, Percival and Ross (8) and Hirst and Rees (4) respectively.

The clear brown syrups which were obtained in all cases, were examined by paper chromatography (solvent 2, spray 1). Strong spots corresponding to 2,3-di-O-methylmannose were observed for all samples, with traces corresponding to mono-O-methyl sugars. Slight traces of faster moving material which might have been tri-O-methyl sugar(s) were also observed, especially in the products from sample 8. Sample 9 also showed a strongish spot with $R_{2,3\text{-di-O-methylmannose}} = 1.5$ and which was not 2,3,4-tri-O-methylmannose. The sugars were converted as described earlier to (a) the mixture of O-acetylated methyl glycosides (b) to the O-acetylated-O-methyl alditols for gas chromatography.

5(b). Examination of the O-acetylated glycosides from the methylated alginates.

Good clean chromatograms were obtained for runs at 175°. The three major peaks corresponded as expected to the derivatives of 2,3-di-O-methylmannose, 2,3-di-O-methylgulose and 1,6-anhydro-2,3-di-O-methylgulose. There were also slower moving substances, presumably mon-O-methyl ethers from undermethylation and/or demethylation, but only in very small amounts. Particular attention was paid to the possible presence of tri-O-methyl sugar derivatives, because if these were present they would indicate chain branching in alginic acid.

Very small amounts (probably less than 0.1%) of 2,3,4-tri-O-methylmannose derivative was observed in samples 1,2,3,4,5,7,9 and 10. At first there seemed the possibility that there might be significant proportions present in samples 6 and 8. Gas-liquid chromatography at lower temperatures revealed that the substance in the samples was different from authentic 2,3,4-tri-O-methylmannose derivative because it had an appreciably faster retention time (15.1cm on the chart at 125°, compared with 15.7cm for the standard).

Very small amounts of derivatives of 2,3,4-tri-O-methylgulose and its 1,6-anhydride appeared to be present in samples 2,3,4,5,9 and 10. The other samples appeared to contain more significant amounts. Gas-liquid chromatography at lower temperatures showed that the peaks in the samples were not due to tri-O-methyl gulose derivatives, however. The two standard substances gave peaks with retention times corresponding to distances on the chart of 1.1 and 4.2cm at 150°, whereas the samples gave only one peak at 1.3cm. Samples 6 and 8 had previously been examined at 125° (see above); comparison of the charts obtained at this temperature with a run of the 1,6-anhydro derivative at the same temperature showed that the peak in the samples could definitely not be attributed to 1,6-anhydride because its retention time corresponded to a distance on the chart of 5.4cm, compared with 3.9cm for the standard.

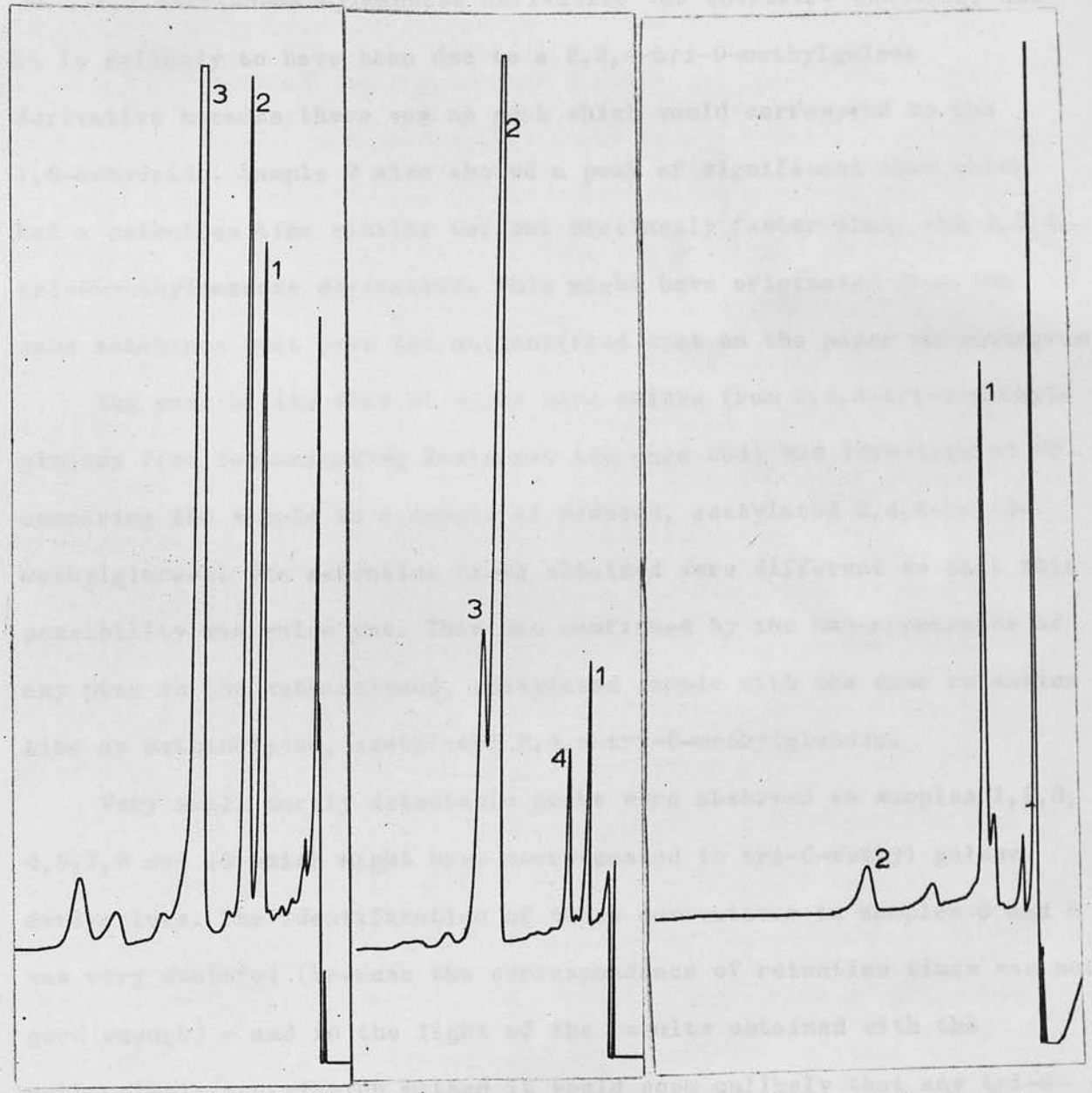
5(c). Examination of the O-acetyl-O-methyl alditols from the methylated alginates.

Good clean chromatograms were again obtained. There was only one extraneous peak, its retention time being given in Table 1. Its retention time was quite different from that of any of the standards and its presence in no way complicated the results.

The major peaks obtained from each sample corresponded to the three di-O-methyl derivatives, with only traces of slower moving substances. The peaks which might have arisen from tri-O-methyl derivatives were again examined carefully.

Very slight traces of a peak that might have corresponded to the 2,3,4-tri-O-methylmannose derivative was observed in all the samples, with possibly up to about 5% in sample 8. The retention time for this peak in the sample corresponded to a distance on the chart of 5.80cm at 175°, compared with 5.95cm for the standard. The assignment of the peak

GAS-LIQUID CHROMATOGRAMS.



methanolysed/acetylated sample	reduced/acetylated sample	reduced/oxidised/reduced/ /acetylated sample
peak 1 = (a)	peak 1 = (a)	peak 1 = (b) + (c)
peak 2 = (b)	peak 2 = (c)	peak 2 = (a)
peak 3 = (c)	peak 3 = (b)	
	peak 4 = (d)	

Key (a) = derivative of 1,6-anhydro-2,3-di-O-methylgulose
(b) = derivative of 2,3-di-O-methylgulose
(c) = derivative of 2,3-di-O-methylmannose
(d) = extraneous peak

Figure 4.

to a 2,3,4-tri-O-methylmannose derivative was therefore doubtful, and it is unlikely to have been due to a 2,3,4-tri-O-methylgulose derivative because there was no peak which would correspond to the 1,6-anhydride. Sample 9 also showed a peak of significant size which had a retention time similar to, but distinctly faster than, the 2,3,4-tri-O-methylmannose derivative. This might have originated from the same substance that gave the unidentified spot on the paper chromatogram.

The possibility that it might have arisen from 2,4,6-tri-O-methyl-glucose from contaminating laminarin (40, page 264) was investigated by comparing the sample to a sample of reduced, acetylated 2,4,6-tri-O-methylglucose. The retention times obtained were different so that this possibility was ruled out. This was confirmed by the non-appearance of any peak in the methanolysed, acetylated sample with the same retention time as methanolysed, acetylated 2,4,6-tri-O-methylglucose.

Very small barely detectable peaks were observed in samples 1,2,3, 4,5,7,9 and 10 which might have corresponded to tri-O-methyl gulose derivatives. The identification of these derivatives in samples 6 and 8 was very doubtful (because the correspondence of retention times was not good enough) - and in the light of the results obtained with the methanolysis/acetylation method it would seem unlikely that any tri-O-methyl derivatives were present.

5(d). Examination of the mixtures of methylated, neutral sugars by the reduction-oxidation-reduction acetylation sequence.

Each solution of methylated sugars (about 20mg) was reduced with potassium borohydride at room temperature overnight, then neutralised (IR-120 resin, H^+ form). The ion exchange resin also removed potassium ions. After evaporation to dryness, methanol was added and the volatile methyl borate removed by evaporation (three additions of

methanol). The residue was dissolved in water and sodium periodate was added. After 16-20 hours, sulphur dioxide was passed through the solution to remove excess periodate and to reduce iodate. The solution was aereated to remove excess sulphur dioxide, then excess potassium borohydride was added until the solution was alkaline. The reduction products were isolated after 16-20 hours with ion exchange resin then methanol in the same manner as previously. Acetylation (acetic anhydride and pyridine for 1 hour at 100°) gave an oily brown product which presumably contained pyridinium salts as well as the desired carbohydrate derivatives. The latter were isolated by extraction with a small volume of chloroform. The extract was evaporated to dryness and methanol was evaporated from the residue (3 times) to remove traces of pyridine. The residue was dissolved in a small volume of chloroform for analysis by gas-liquid chromatography.

All samples showed a major peak corresponding in retention time to 1,4-di-O-acetyl-2,3-di-O-methylerythritol. No peaks were observed in any sample which might have corresponded to the acetates of 2,3-di-O-methylthreitol or 2,4-di-O-methylarabitol. There were several minor peaks which probably corresponded to other periodate oxidation products and/or their condensation products, and also a peak which corresponded to the acetate of 1,6-anhydro-2,3-di-O-methylgulose, which arose because any 2,3-di-O-methylgulose which was present in the mixture of neutral sugars as its 1,6-anhydride would survive the reduction and oxidation steps unchanged.

The volatility of 1,4-di-O-acetyl-2,3-di-O-methylerythritol has already been noted (Experiment 3). This was confirmed by the finding that the products from sample 4, after standing several days in a stoppered test tube, gave the same peaks as originally but with the

proportion of the erythritol derivative considerably reduced.

DISCUSSION.

The complete absence of the acetates of 2,3-di-O-methylthreitol and 2,4-di-O-methylarabitol from the products of the reduction/oxidation/reduction/acetylation sequence of reactions suggests that alginic acid contains only 1,4 linkages. The other two series of reactions, which acted as a cross-check on one another, confirm that the only hydrolysis products of methylated alginic acid are the 2,3-di-O-methyl ethers of mannuronic acid and guluronic acid, and in particular that there are, at the most, only minute quantities of tri-O-methyl ethers. The molecule would therefore appear to be unbranched. These conclusions hold for alginates from several different seaweed species, from stipes, from old and young fronds, and with varying mannuronic acid/guluronic acid ratios.

It is noteworthy that for Samples 1,2,3 and 4, whose mannuronic acid/guluronic acid ratios are known, the ration of the derivatives of these acids was found to be, by inspection of the gas-liquid chromatograms, proportional to the ratio of the uronic acids. By this criterion it was observed that the sample which had previously been investigated by Chanda et.al.(8), was in fact a guluronic acid-rich alginate.

Sample 8 was of special interest because it had been subjected to prolonged periodate oxidation before methylation. The results have shown that the periodate-resistant units are 1,4-linked. It is presumed that steric factors, perhaps related to the secondary structure of the polysaccharide, are responsible for their failure to react even in solution.

SECTION B.

CARBOXYL REDUCTIONS OF ALGINIC ACID.

INTRODUCTION.

Alginic acid which contains only uronic acid units, is in many ways difficult to work with and reduction of the molecule to give a neutral polysaccharide affords a much more amenable starting material for structural studies. For example, the neutral polysaccharide is considerably more amenable to partial acid hydrolysis than alginic acid itself and furthermore the derived neutral oligosaccharides are more readily separated by standard chromatographic techniques than the corresponding oligouronic acids. However, no absolutely general method for the reduction of acidic polysaccharides was available and accordingly such a method was the object of the present section.

Although the reduction of most carboxylic acids and esters to the corresponding primary alcohols can be carried out conveniently and quantitatively by the use of suitable hydrides, such reactions are not suitable for polysaccharides (41). These substances are insoluble in the solvents normally used and although their methyl ethers are often soluble and can be reduced, the products cannot be recovered in the unsubstituted form. Aqueous media can be used when the ester is reduced with sodium or potassium borohydride, but ester hydrolysis also occurs and it is necessary to repeat the esterification and reduction several times. Heterogeneous reduction (42) is usually incomplete because not all the molecules in the solid phase are accessible to the reagent.

Another method (42) that has been successful in some instances, is to make the polysaccharide soluble in the reaction solvent by acetylation or propionylation and then to reduce the carboxylic acid groups with diborane; the solubilising groups can be removed afterwards.

Unfortunately the diborane reduction of alginic acid is incomplete (42,9) and a highly undesirable side-reaction occurs under the best conditions for rapid reaction (9).

There were then two possible approaches to a reduction procedure. The first method attempted was the reduction of a soluble derivative of alginic acid. This was based on the observation (43) that tri-methyl silyl ethers react very slowly with lithium aluminium hydride so that reduction of the carboxyl groups might proceed without appreciable reduction of the solubilising tri-methyl silyl ether groups. The ether groups could then be removed after reduction of the carboxylic groups was complete. The problem in this case was therefore one of obtaining a soluble silylated derivative of alginic acid.

The second alternative was to effect a heterogeneous reduction on a derivative of alginic acid in which all the molecules were amenable to the reducing agent.

After completion of the work described in this section a critical assessment (44) of the effects of three different solvents (water, 80% aqueous dimethyl sulphoxide, and 80% aqueous methanol) on the efficiency of carboxyl reductions by potassium borohydride on the methyl and ethylene glycol esters of sunflower pectic acid was published. It was found that the best procedure for reduction of pectic acid methyl ester is a repeat process of heterogeneous esterification with diazomethane followed by borohydride reduction in 80% aqueous methanol, until the product contains less than 20% of unreduced galacturonic acid. At this stage, both esterification and reduction can be carried out in 80% aqueous dimethyl sulphoxide to give a fully reduced product. The authors reported a fall in the degree of polymerisation from 270 for the original polysaccharide to 21 for the fully methylated reduced

product and claimed that this was due to the reduction procedure. However it seems quite likely that the degradation may quite well have been due in part, if not wholly, to the diazomethane treatment of the acid (45) and/or exposure to extreme pH during methylation of the reduced polysaccharide.

EXPERIMENTAL.

Experiment 6. Complete carboxyl reduction of a soluble derivative of alginic acid and detection of the artefacts formed in the process.

6(a). Complete carboxyl reduction of alginic acid by conversion to the methyl ester with diazomethane, followed by heterogeneous trimethylsilylation and reduction of the soluble product with lithium aluminium hydride in tetrahydrofuran.

Alginic acid was prepared from a commercial sample of sodium alginate (Manucol), and while still wet it was washed three times with methanol and finally suspended in methanol. Ethereal diazomethane was added until the supernatant solution remained yellow for at least half an hour. After leaving in an open flask overnight to allow the diazomethane to evaporate, water was added and then the solution was evaporated to a small volume to remove the methanol. Addition of hydrochloric acid to a small part of the solution gave no precipitate; the solution was passed through IR-120 resin to convert any residual uronic acid units to the acid form. Esterification was evidently incomplete because the effluent had pH about 3. The solution was therefore freeze-dried and the product was suspended in methanol and again treated with diazomethane. An overall yield of 49% was obtained of the methyl ester. Later experiments (see experiment 9(c)) suggested that the commercial sodium alginate contains large amounts of inorganic contaminants (? silica); the presence of these would account for the low yield.

Methyl ester (50mg) was added to anhydrous pyridine (5ml) and hexamethyldisilazane (1.0ml) was added with trimethylchlorosilane (0.5ml) (46). The mixture was shaken for 30 minutes. The polysaccharide dissolved, and simultaneously a cloudy precipitate (presumably

ammonium chloride) was observed. The solvent and excess reagents were removed under vacuum at room temperature, and the last traces of pyridine were removed by the repeated evaporation of anhydrous carbon tetrachloride under vacuum. The residue was dissolved in tetrahydrofuran (10ml), and chilled in an ice bath. Lithium aluminium hydride (50mg) was added in tetrahydrofuran solution (10ml), over a period of 10 minutes. The mixture was left at room temperature overnight. Excess of hydride was destroyed by the addition of ethyl acetate, and water was added until the precipitation of lithium aluminate was complete. The solution was evaporated to dryness and the residue hydrolysed with 50% formic acid at 100° overnight. The aqueous solution of hydrolysed products was treated with IR-120 resin to remove Li^{+} and Al^{+++} ions. Electrophoresis showed the hydrolysate to consist almost entirely of neutral sugars. Paper chromatography (solvent 1) showed only traces of uronic acid. Four spots appeared on the sprayed chromatogram, however. The heaviest of these corresponded to mannose plus gulose. The two fastest-moving spots were shown to be the mono- and diformyl esters of the neutral sugars by comparing them with the mixture of products obtained by treatment of mannose with 50% formic acid at 100° overnight. On further hydrolysis of the products from the reduced polysaccharide with 0.1N sulphuric acid at 100° overnight, these two spots disappeared. A spot was intermediate in position between the mannose plus gulose spot and the monoformate spot and this did not disappear as a result of the further acid hydrolysis. Further tests confirmed the identity of this spot.

6(b). Detection of the methyl ethers formed in the esterification stage of the reduction process.

Evidence that the unknown spot mentioned in section (a) was due to

the presence of monomethyl sugars was:-

(i) Hydrolysis and paper chromatography of the polysaccharide methyl ester gave spots corresponding to the uronic acids and the corresponding lactones. These same spots were given by sodium alginate after hydrolysis. In addition however, the hydrolysate of the methyl ester gave spots that were not given by the hydrolysate of the sodium salt. These two new spots travelled (a) slightly faster than the uronic acids and (b) slightly faster than the lactones. Their R_F values and the fact that they were present only after diazomethane treatment and were acid stable suggested that they were methyl ethers.

(ii) The presumed methyl ether in the hydrolysate had the same R_F value as 4-O-methylmannose. It is likely that all the mono-O-methyl ethers would travel at the same rate in solvent 1, and the unknown spot could therefore correspond to a mixture of mono-O-methyl mannoses and guloses.

(iii) Treatment of the reduced polysaccharide with boron trichloride (47) followed by paper chromatography gave only mannose and gulose. Methyl ethers, if present would have been cleaved by the boron trichloride.

(iv) Previous workers (48) have shown that even brief exposure of alginic acid to diazomethane causes the introduction of some methyl ether groups (Zeisel determination) that cannot be removed with alkali and are therefore presumably ether groups.

Experiment 7. Attempted preparation of methyl alginate.

Since the formation of methyl ether artefacts was regarded as a highly undesirable side-reaction in the reduction process described in experiment 6, attention was turned to preparation of a fully methyl-esterified alginate containing no methyl ether groups. The criterion used to estimate the extent of methyl esterification was silylation of

the product and treatment with lithium aluminium hydride as before, followed by hydrolysis and paper chromatography and electrophoresis. The extent of reduction was taken as a measure of the esterification. The following esterification procedures were attempted:-

(i) A solution of sodium alginate was passed through a column of IR-120 resin (NH_4^+ form) in order to convert it to ammonium alginate. The solution was concentrated and freeze-dried and the residue was treated with ethereal diazomethane (3 hours) (49). Reduction was unsuccessful and it would therefore appear that the esterification was very incomplete.

(ii) Alginic acid (freshly prepared) was treated with ethereal diazomethane for 10 minutes only. Esterification was again incomplete.

(iii) Freeze-dried alginic acid was suspended in ether : methanol (10 : 1) and treated with sufficient ethereal diazomethane to give a persistent yellow colour, for 2 minutes, 10 minutes, 1 hour and 24 hours.

An attempt was then made to fractionate the polysaccharide into esterified and non-esterified fractions by extracting the product with water and discarding any residue. The amount of such residue decreased with increasing period of exposure to diazomethane; consistently with this, the weight of ester isolated by subsequent freeze-drying of the aqueous extract increased. The "ester fractions" appeared to be completely esterified, because they could be completely reduced. However methyl ethers were observed as artefacts in all the reduction products.

(iv) Alginic acid was dissolved in dimethylsulphoxide and sufficient ethereal diazomethane was added to give a persistent yellow colour, but not to precipitate the polysaccharide. After periods of 2 minutes, 10 minutes, 1 hour and 24 hours, the polysaccharide was precipitated

by the addition of a large excess of acetone. The product was fractionated into "ester" and "acid" fractions by solution in water as in (iii) above. The "ester" fraction, even those isolated after prolonged diazomethane treatment, were incompletely esterified as judged by the reduction criterion. N.B. The validity of this criterion is considered in the discussion.

(v) The experiment described under (iv) was repeated except that methanol (50) was added to act as a catalyst in the esterification. There was no improvement.

(vi) The 2-hydroxyethyl ester of alginic acid (see General Methods) was treated with 1% methanolic hydrogen chloride overnight to attempt to prepare the methyl ester by transesterification. The product, which proved to be largely insoluble in water was freeze-dried. No reduction was achieved.

(vii) An attempt at acid-catalysed transesterification in a homogeneous system (dimethylsulphoxide-methanol, in the presence of hydrochloric acid), was likewise unsuccessful.

(viii) The 2-hydroxyethyl ester was shaken with sodium methoxide (0.015N) for varying periods (1 hour and 2 days). The products were dissolved in water and freeze-dried. Reduction was again unsuccessful.

(ix) Alginic acid (0.10g) was suspended in dry methanol (10ml) and then trifluoroacetic anhydride (51,52) was added (0.20ml) and the mixture was shaken for 2 days. The residue was isolated by decantation and washed with acetone. It did not seem to be very soluble in water and was therefore freeze-dried as a suspension. An attempt to reduce the residue in the usual way was unsuccessful. In a second attempt to achieve esterification through the mixed anhydride in this way the reaction was carried out in the presence of some trifluoroacetic acid

as well as the anhydride. The object of adding the acid was to swell the polysaccharide. The experiment was also unsuccessful, however.

Experiment 8. Attempted homogeneous reduction of a mixed ester of alginic acid using the benzyldimethylsilyl group as a solubilising group.

In an attempt to effect a homogeneous reduction of alginic acid it was decided firstly to form a mixed ester, using a mixture of epoxides for the esterification, in the hope that a more soluble product might be obtained, in the same way as esterification with a mixture of acids gives a more soluble derivative than with a single acid in the esterification of other polysaccharides (53). Secondly, it was decided to use the benzyldimethylsilyl group as a solubilising group since this might confer more solubility in the pyridine solvent than the trimethylsilyl group which was used in experiment 6(a).

A mixed ester of alginic acid (MCI) was prepared using ethylene oxide and propylene oxide as the esterifying agents. Mixed ester (50mg) was shaken with pyridine (10ml) for 1 hour, then benzyldimethylchlorosilane (1ml) was added and shaken at room temperature for 1 hour. The carbohydrate appeared to be soluble, but some precipitate was present. This was shown to be pyridinium chloride since on gentle heating, the precipitate became completely soluble but reappeared as fine needles on cooling. Hexamethyldisilazane (2ml) was added, giving a white precipitate, presumably of ammonium chloride. The solvent and excess reagents were removed under vacuum and the last traces of pyridine were removed by the repeated evaporation of anhydrous carbon tetrachloride under vacuum. Theresidue was dissolved in tetrahydrofuran (10ml), giving a milky solution, and lithium borohydride (50mg) was added in tetrahydrofuran solution (10ml). The mixture was

refluxed for 20 hours, then ethyl acetate was added to destroy the excess lithium borohydride. Water was added and the tetrahydrofuran was then removed by concentrating the solution to a small volume. At this stage an oily precipitate appeared in the aqueous solution and was shown to give a strong reaction with the phenol/sulphuric acid reagent, whereas the aqueous solution itself only gave a weak reaction. Evidently the oil was silylated polysaccharide. To effect desilylation, the solution was made up to 50% aqueous methanol (54) and was refluxed for 16 hours, but oily material remained. The methanol was evaporated by concentrating the solution to a small volume. Extraction of benzyldimethylsilanol was then attempted with an equal volume of benzene. The benzene layer gave a stronger positive reaction to the phenol/sulphuric acid reagent than the aqueous layer did, indicating that the polysaccharide had not been desilylated to any extent. The benzene was then evaporated and to the aqueous solution was added potassium borohydride (10mg). After standing overnight, the flask was flushed with nitrogen and then the solution was made to 0.5N with respect to sodium hydroxide. After shaking for 70 hours the black oily material was completely solubilised and on extraction with benzene the carbohydrate was shown to be wholly present in the aqueous layer. The aqueous extract was dialysed. Hydrolysis and paper chromatography and paper electrophoresis of a sample of the dialysate showed neutral sugars and uronic acids in the approximate ratio 3 : 1.

Examination of the mixed ester starting material by the deuteration/infra-red technique (see experiment 11(b)) indicated that the sample was incompletely esterified and perhaps this could account for the incomplete reduction by the above method.

It is noteworthy that when the experiment was repeated using

lithium aluminium hydride, the same degree of reduction was obtained. Essentially the same procedure was used in this experiment and it was found that after refluxing with lithium aluminium hydride a greater degree of desilylation appeared to have taken place than at the corresponding stage of reaction with lithium borohydride.

Experiment 9. Heterogeneous carboxyl reduction of esters of alginic acid and some derivatives of these esters.

9(a) Heterogeneous carboxyl reduction of esters of alginic acid.

Preliminary experiments showed that the reduction of methyl di-O-trimethylsilylalginate (containing some methyl ether groups as discussed in experiment 6(b)) could be carried out just as effectively with lithium borohydride in boiling tetrahydrofuran for 16 hours, as with lithium aluminium hydride at a lower temperature. It was also found that the same treatment with lithium borohydride would reduce the non-trimethylsilylated ester to the same degree, (although lithium aluminium hydride would not) even though the reaction was heterogeneous.

The 2-hydroxyethyl and 2-hydroxypropyl esters (prepared from Manucol) could not be completely reduced in this way (the proportion of units that were reduced was estimated at about 60% simply by the inspection of paper chromatograms after hydrolysis). A second heterogeneous reduction of the partially reduced 2-hydroxyethyl ester isolated by freeze-drying, gave no further reduction. Both mannose and gulose were obtained after hydrolysis of the partially reduced product (solvent 8). An attempt to carry out the reduction of the 2-hydroxyethyl alginate homogeneously in a mixture of formamide and tetrahydrofuran was unsuccessful, as it appeared that the formamide was reduced preferentially.

9(b) Preparation and heterogeneous reduction of methyl di-O-acetyl-alginate.

Complete reduction was obtained without any artefact formation, by the heterogeneous reduction of methyl di-O-acetylalginate with lithium borohydride in boiling tetrahydrofuran for 16 hours. This derivative was prepared from sodium alginate (Manucol) via the alginic acid, which was then acetylated in the "activated" form with acetic anhydride in acetic acid using perchloric acid as catalyst (55), and then treated with diazomethane. The overall yield would appear to be poor (26%), but it was found later that the sample of sodium alginate contained large amounts of inorganic contaminant (? silica) that would depress the yield (see experiment 9(c)). After the preparative scale reduction of the methyl di-O-acetylalginate (0.375g), the product (0.168g = 76%) was isolated by dialysis and freeze-drying. It showed no carbonyl peaks in the infra-red, was freely soluble in water and showed barely detectable traces of uronic acids on paper chromatography and paper electrophoresis after hydrolysis. Uronic anhydride determinations (by decarboxylation and titration) gave a value of 4.7% compared with 3.5% that has been reported for mannose (56)

9(c) Preparation and heterogeneous reduction of 2-acetoxyethyl di-O-acetylalginate.

Following on from the success of the reduction method described in experiment 9(b), it was decided to attempt the reduction of the acetate of the 2-hydroxyethyl ester of alginic acid. An attempt to acetylate the ester with pyridine and acetic anhydride was unsuccessful as the alginate remained insoluble even after prolonged heating in a water bath. N-Methyl 2-pyrrolidone and dimethylsulphoxide were both examined as possible reaction solvents. N-Methyl 2-pyrrolidone

proved the more suitable, because less darkening of the reaction mixture was observed in this solvent (57). The following method was therefore used for the acetylation of the 2-hydroxyethyl ester of alginic acid:-

2-Hydroxyethyl ester (100mg) was partially dissolved with heating in N-methyl 2-pyrrolidone (8ml). Acetic anhydride (3.5ml) and pyridine (12ml) were then added over 15 minutes with heating and shaking so that all the carbohydrate was soluble. After heating at 100° for 45 minutes, the solution was poured into a large excess of water to give a precipitate of the acetylated product. After dialysis, the solution was evaporated to a small volume and the acetylated product was extracted with chloroform. The acetylated product was then precipitated with light petroleum, filtered and dried in a vacuum oven.

After dialysis of the acetylated product followed by chloroform extraction, evaporation of the aqueous layer gave a residue (36% of the original weight) which gave a negative phenol/sulphuric acid test for carbohydrate. The material gave no infra-red spectrum, and gave large amounts of a glassy solid on combustion (ash content about 40%). Uronic anhydride (decarboxylation and titration) determination on the original sodium alginate gave a percentage purity of 68%, confirming that this commercial sample was grossly contaminated with some other material (? silica). The chloroform-soluble fraction gave no hydroxyl peak in the infra-red. Heterogeneous reduction with lithium borohydride in boiling tetrahydrofuran for 16 hours, followed by dialysis and freeze-drying gave a product (68% yield as calculated from uncontaminated ester) that was water soluble and showed no carbonyl absorption in the infra-red. Hydrolysis and paper chromatography and

paper electrophoresis showed that detectable amounts of uronic acid (less than 5%) were present.

Experiment 10. Other attempts at carboxyl reduction.

10(a) Other attempts at carboxyl reduction of alginic acid.

Treatment of sodium alginate, alginic acid and methyl alginate (partly methyl etherified) with lithium aluminium hydride in tetrahydrofuran overnight at room temperature gave no reduction. There was no reduction when alginic acid was trimethylsilylated and treated with lithium aluminium hydride. There was no reduction when 2-hydroxyethyl alginate, before or after treatment with ethereal diazomethane, was similarly treated. A further attempt to obtain a soluble trimethylsilyl ether of 2-hydroxyethyl alginate was made by heating it to 110° in formamide (58), evaporation of most of the formamide, and then treatment with the usual agents for trimethylsilylation. Subsequent treatment of the product with lithium aluminium hydride in tetrahydrofuran gave no reduction. The 2-hydroxypropyl and 2-hydroxy-2-phenylethyl alginates were likewise not reduced when treated with the reagents for trimethylsilylation followed by lithium aluminium hydride reduction. The 2-hydroxyethyl ester was only partly reduced by sodium borohydride in dry methanol at room temperature for 48 hours.

10(b) Carboxyl reduction of other polysaccharides.

Little if any reduction (as judged by hydrolysis and paper chromatography and paper electrophoresis) was obtained of the 2-hydroxyethyl esters of Escherichia coli (S 53) slime polysaccharide (59) and of the mixture of pectic polysaccharides from white mustard (60) when they were treated with the reagents for trimethylsilylation followed by lithium aluminium hydride reduction. In contrast, heterogeneous reduction with lithium borohydride of the acetylated

2-hydroxyethyl ester of the mustard polysaccharide, gave almost complete reduction.

Experiment 11. Attempts to relate the accessibility of esterified alginates to reduction and other reactions.

It has been suggested (13) that the linear alginate molecule consists of "blocks" with either predominantly mannuronic or guluronic acids and that these "blocks" are separated by regions with another sequence of uronic acid residues, probably with a large proportion of alternating mannuronic and guluronic acid residues. The difference in hydrolysability of these regions has been attributed to a difference in crystallinity; the blocks with a highly regular structure more easily forming crystalline regions in the insoluble state, and those highly ordered regions having a much lower rate of hydrolysis than the more amorphous regions.

Results of heterogeneous reductions (experiment 9(a) and 10(a)) on glycol esters of alginic acid suggest that this crystallinity factor might be limiting the amount of reduction. Accordingly it was thought that diazomethane treatment of these esters might result in disruption of the crystalline regions by the formation of methyl ethers so that complete reduction might be obtained. It was also hoped to be able to estimate the degree of crystallinity in various alginate samples by the deuteration/infra-red spectroscopy technique (61).

11(a). Heterogeneous reduction of diazomethane-treated 2-hydroxyethyl alginate.

The ester (20mg) was treated with ethereal diazomethane containing 10% methanol for 3 days at room temperature. After evaporation of excess diazomethane together with most of the ether and methanol, the product dissolved readily in water and was freeze-dried.

It was then refluxed with an equal weight of lithium borohydride in tetrahydrofuran for 16 hours, and treated with ethyl acetate. After evaporation to a small volume, water was added to dissolve the residue and the solution was dialysed. Formic acid hydrolysis followed by electrophoresis and paper chromatography (solvent 1) showed substantial amounts of uronic acid, traces of mannose and/or gulose and several strong spots which were fast-moving on paper chromatograms. The first of these corresponded to the methyl ether previously reported (experiment 6(b)). The faster spots corresponded to formyl esters and some lactone.

11(b) Deuteration/infra-red spectroscopy of alginate samples.

Changes in the spectra during deuteration did not show the appearance of any sharp hydroxyl bands, as in the case of cellulose (61), in the following alginates:-

L. cloustonii alginate (2-hydroxypropyl ester), L. cloustonii alginate (2-hydroxyethyl ester), L. cloustonii alginate (heterogeneously reduced 2-hydroxypropyl ester), L. cloustonii alginate (twice heterogeneously reduced 2-hydroxypropyl ester), L. digitata alginate (MCI 2-hydroxyethyl ester), L. digitata alginate (Manucol 2-hydroxyethyl ester). This technique was therefore unsuitable.

DISCUSSION.

Attempts to form then reduce a methyl ester of alginic acid, free from methyl ethers, were unsuccessful. It is not known whether this was owing to failure at the esterification or at the reduction stage. It was assumed that the amount of reduced material obtained after silylation and reduction with lithium aluminium hydride, was proportional to the amount of methyl-esterified material at the start. In retrospect this may not have been a true criterion since a completely or partly esterified polysaccharide may not reduce at all owing to physical factors, which were shown to be important by subsequent work. For example, the successful homogeneous reduction of the partly methyl-etherified methyl ester of alginic acid by lithium aluminium hydride would then be attributed at least partly to the presence of the methyl ether groups in the polysaccharide. It would appear that these groups are suitably placed to prevent tight association (and hence inaccessibility to the trimethylsilylating reagents) in the crystalline regions of the solid state. The observation that diazomethane causes β-elimination in esterified polysaccharides (45) points to another possible reason why successful reduction is obtained. Smaller fragments of polysaccharide chains would be more easily solubilised to give a reducible product. The possible presence of crystalline regions is discussed in section D.

The attempt to reduce a mixed ester of alginic acid homogeneously, using benzyldimethylchlorosilane as a solubilising agent met with partial success. As has already been stated, the starting material was found to be incompletely esterified and perhaps this would account for the incomplete reduction. This experiment will have to be repeated on a fully esterified sample to see whether complete reduction is

obtainable by this method. Two interesting points do arise out of the method however. Firstly it gives a method of obtaining a soluble polysaccharide derivative and secondly the fact that carboxyl reduction is obtained without any apparent degree of silyl ether reduction may be of use in structural studies, since the silyl ether groups could serve as blocking and solubilising groups, leaving the primary hydroxyl group free for further reactions.

Although other attempts to produce a soluble trimethylsilyl derivative of alginic acid were unsuccessful, some interesting light was shed on the chemistry of alginic acid. As already stated, the successful reduction of the partly-etherified methyl ester of alginic acid is probably at least partly due to the disruption of the crystalline regions of the molecule by methyl ether groups. The slowness of heterogeneous esterification of alginic acid by diazomethane could also be due to inaccessibility effects of this sort. After short periods of reaction, fully esterified (as shown by the fact that it could be completely reduced) alginic acid could be isolated by extraction with water. It would appear that such molecules are completely amorphous. The material that was insoluble in water presumably contained amorphous and crystalline regions and would (in the early stages of reaction at least) be esterified in the amorphous regions only. It is noteworthy that diazomethane can react in non-crystalline and imperfectly crystalline regions of cellulose; but not in the highly ordered crystalline regions (62). Even in solution in dimethylsulphoxide the reaction between alginic acid and diazomethane is incomplete. This would suggest that there is some inter- or intramolecular bonding even in solution and this hinders esterification. This might be related to the periodate resistant

fraction of soluble sodium alginate (see section A).

The failure of glycol esters to give soluble trimethylsilyl ethers is again presumably due to inaccessibility factors. X-ray diffraction gave qualitative indications that regions of crystallinity do exist in these glycol esters.

The fact that the partly-etherified methyl ester of alginic acid can be heterogeneously reduced to almost the same degree as by the trimethylsilylation procedure, by simply refluxing with lithium borohydride, coupled with the fact that glycol esters are only partly reduced this way, again suggests that inaccessibility factors are hindering complete reduction of the glycol ester. Virtually complete heterogeneous reduction was obtained with the methyl ester of the acetate of alginic acid and also with the acetate of 2-hydroxyethyl alginate. It would appear that these reductions were successful because the reductive removal of acetate groups led to a wide open structure through which the reducing agent could penetrate.

The question has been raised as to whether much degradation of the polysaccharide accompanies these reduction procedures (44). It has been shown in this department that reduction of Araucaria bidwelli gum (63) via the acetate of the glycol ester does produce a lower molecular weight product as judged by ultracentrifugation, but that degradation is not extensive (64).

The ineffectiveness of lithium aluminium hydride in heterogeneous reductions can be attributed to its affinity to complex with hydroxyl groups, so that a protective film is formed about each particle providing resistance to any further reduction.

Experiment 11(a) was designed to correlate the accessibility/crystallinity problem. The idea was that the amorphous regions of the

ester would be partly etherified by treatment with diazomethane, so that on dissolving and freeze-drying this product, there would be a minimum of crystallinity and complete reduction with lithium borohydride might be obtained. Ethers of mannose and gulose were observed, but only traces of mannose and gulose themselves; uronic acid was chiefly in the non-methylated state. It would therefore appear that the regions accessible to diazomethane are readily reduced and that the non-reduced parts are those which were also inaccessible to diazomethane. This suggests that these regions that are crystalline reappear as crystalline regions after dissolving and freeze-drying.

SECTION C.

BASE-CATALYSED DEGRADATION OF ALGINIC ACID.

INTRODUCTION.

The alkaline degradation of oligo- and polysaccharides has received increased attention in recent years. It is now generally accepted that alkaline degradation of 1,3-or 1,4-linked polymers of neutral sugar units, in the absence of oxygen, proceeds from the reducing end-groups by a stepwise elimination of monosaccharide residues (65). The latter are transformed into saccharinic acids and acids of lower molecular weight as has been shown by Richards and Sephton (66). However, reactions very rarely proceed to completion. For example, in the degradation of cellulose an occasional elimination of a 3-hydroxyl group rather than a 4-glycosyl-oxy group will give a terminal metasaccharinic acid which blocks any further reaction. Polysaccharide chains are much more rapidly and extensively fragmented by alkali after oxidation with periodate and some other reagents. On the other hand, it is noteworthy that the glycosidic linkages within the chains are normally extremely alkali resistant if the polysaccharide chain has no reducing end-group.

Neukom and Deuel (45) have found that pectin - the partial methyl ester of pectic acid - behaves like an oxidised polysaccharide, that is, glycosidic linkages within the chain are readily split by alkali, producing fragments of lower molecular weight. They also observed the same phenomona with glycol esters of pectic and alginic acids and also claimed that non-esterified material was alkali stable. They suggested that the instability of esters of alginic acid and pectic acid could best be explained by assuming an elimination reaction to form a double bond in the α,β-position to the carbonyl group.

According to this mechanism the glycosidic linkage in the β-position to the ester carbonyl group is cleaved following the removal of the activated hydrogen at C-5 and the formation of a double bond between C-4 and C-5 as shown in figure 5. This theory has since been substantiated by Neukom and co-workers (67,68) by work on model compounds. They prepared and characterised the α,β-unsaturated methyl ester of the α-methyl glycoside of galacturonic acid by β-elimination reactions on two different starting compounds. They were able to obtain this product from both the methyl ester of α-methyl galacturoniside and the dimethyl ester of α-methyl digalacturoniside by the action of sodium methoxide in methanol.

Since the proof of structure by Neukom and his collaborators, various workers have shown the β-elimination reaction to take place, both chemically and enzymically, in uronic acid-containing polysaccharides.

Albershiem, Neukom and Deuel (69) have shown that pectin breaks down rapidly by heating in aqueous buffer at pH 6.8 and claimed that degradation is dependent on the presence of the ester. Their results were consistent with the formation of an unsaturated compound as in the β-elimination reaction. They also showed that continued degradation gave rise to unsaturated compounds of increased conjugation as judged by ultra-violet spectra. Haug, Larsen and Smidsrod (19), investigating the degradation of non-esterified alginates at different pH values, have shown that even in neutral and slightly acid media a significant part of the degradation occurs as β-elimination. They used the sensitive thiobarbituric acid test (70) as the criterion for the β-elimination reaction and were thus able to show that the reaction could be activated by non-esterified alginate. Barrett and Northcote (71),

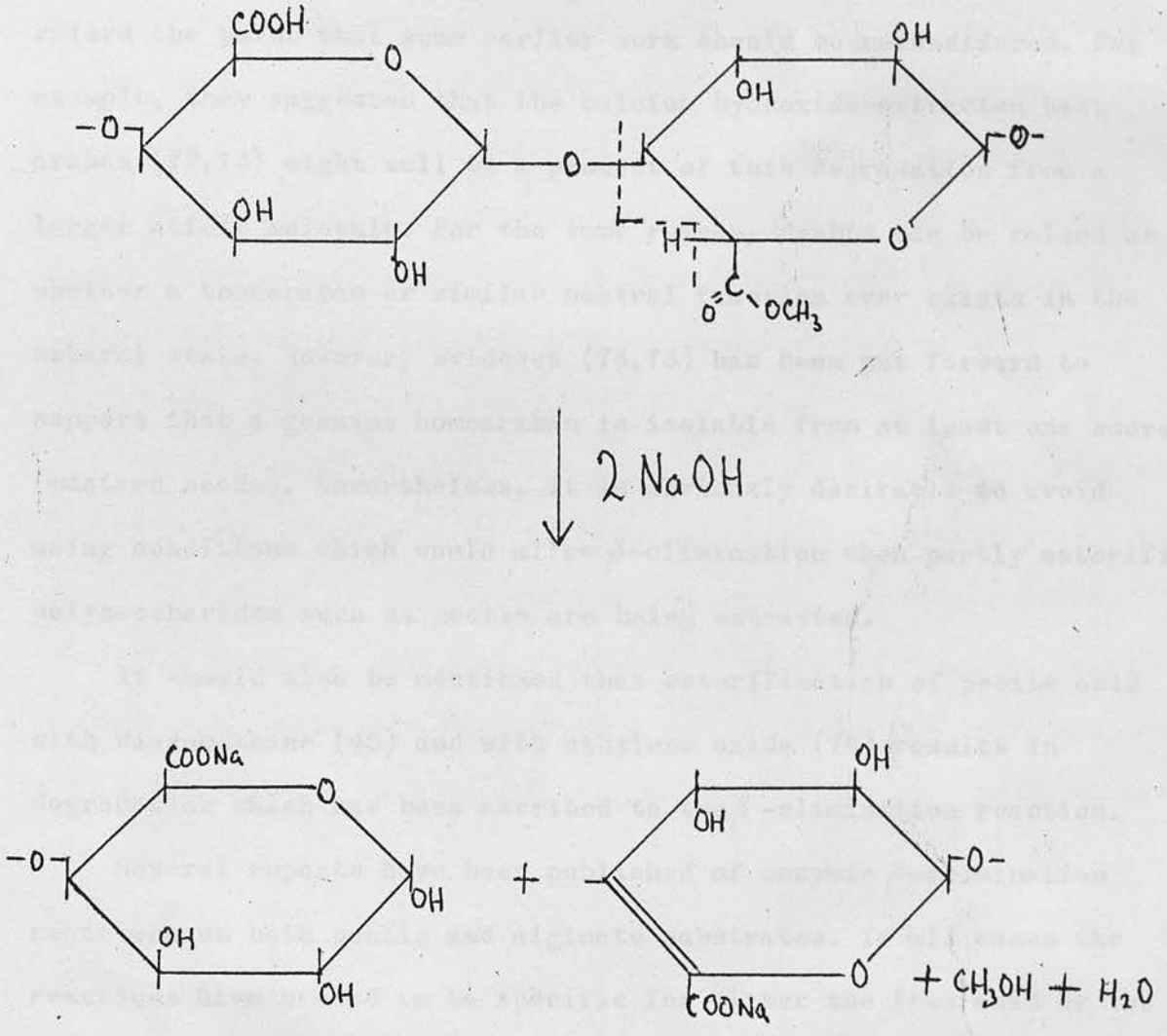

COOH
O
OH
OH
OH
OH
O
H
O
O
C
O
OCH3
2 NaOH
COONa
O
OH
OH
OH
OH
O
O
+
+ CH3OH + H2O
COONa

Figure 5.

utilising the β-elimination reaction on pectin, showed that the degradation obtained by heating at pH 6.8 gave rise to a fraction consisting mostly of uronic acid units and one containing mostly neutral sugar units. In isolating the neutral fraction; the authors raised the point that some earlier work should be reconsidered. For example, they suggested that the calcium hydroxide-extracted beet araban (72,73) might well be a product of this degradation from a larger acidic molecule. For the same reason, doubts can be raised as to whether a homoaraban or similar neutral fraction ever exists in the natural state. However, evidence (74,75) has been put forward to support that a genuine homoaraban is isolable from at least one source (mustard seeds). Nevertheless, it is obviously desirable to avoid using conditions which would allow β-elimination when partly esterified polysaccharides such as pectin are being extracted.

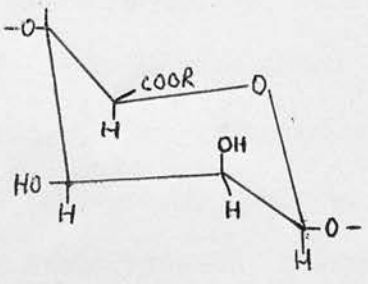
It should also be mentioned that esterification of pectic acid with diazomethane (45) and with ethylene oxide (76) results in degradation which has been ascribed to the β-elimination reaction.

Several reports have been published of enzymic β-elimination reactions on both pectic and alginate substrates. In all cases the reactions have proved to be specific for either the free acid or the ester but not for both. A bacterial polygalacturonic acid-trans-eliminase has been isolated from Clostridium multifermans which has been shown to remove units of α,β-unsaturated digalacturonic acid from the reducing ends of polygalacturonate chains (77). This enzyme did not attack fully esterified pectin. A polygalacturonic acid-trans-eliminase which randomly degrades the polygalacturonate chains has been isolated from Bacillus polymyxa (78). In the case of alginic acid an enzyme fraction referred to as alginase has been reported to

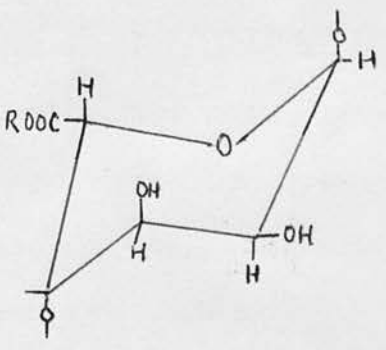
cleave alginic acid into a series of oligosaccharides containing an unsaturated uronic acid on the non-reducing end of the oligosaccharide chain (17). Further degradation of the unsaturated oligosaccharides by the enzyme ultimately gave the monosaccharide, the α-keto acid, 4-deoxy-L-erythro-5-hexoseuloseuronic acid (see figure 10). Two separate alginic acid eliminases have been purified from abalone hepatopancreas (18). The first of these, referred to as alginase I, was specific for β-1,4 bonds involving mannuronic acid units and appeared to act at random on internal bonds. Alginase II was reported to be specific for 1,4 bonds involving guluronic acid and appeared to work at or near chain ends.

As regards the possible scope for structural studies by a chemical β-elimination procedure, the reaction might well be utilised on both alginic acid and pectin. If the reaction should prove to be stereo-specific, requiring trans leaving groups, then β-elimination would occur in guluronic acid residues and not in mannuronic acid residues in alginic acid so that selective cleavage in the molecule might be obtained.

Figure 6.



Ester of mannuronic acid.

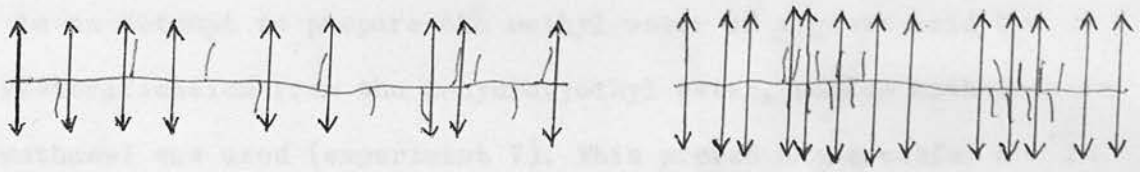


Ester of guluronic acid.

In the case of fully esterified pectin the β-elimination reaction can be used to determine to a certain extent the side chain distribution along the polygalacturonate main chain. If it happens to be random, then random cleavage by β-elimination would result in a single molecular weight distribution curve, whereas if there are blocks with side chains

and blocks without, then a distribution of acidic fragments and a distribution of neutral fragments would result after β-elimination and de-esterification. This is illustrated in the following diagrams.

Figure 7.



(A) Random distribution of side chains (B) Block distribution of side chains.

EXPERIMENTAL.

Experiment 12. Fragmentation of esters of alginic acid by base-catalysed β-elimination with the formation of 4,5-unsaturated products and evidence for further reactions.

In an attempt to prepare the methyl ester of alginic acid by transesterification from the 2-hydroxyethyl ester, sodium methoxide in dry methanol was used (experiment 7). This proved unsuccessful and it was thought that perhaps traces of water in the system might have inhibited the reaction by causing de-esterification and consumption of base. It was therefore decided to add to the system some 2,2-dimethoxypropane which is known to be a water "scavenger". It was also kept in mind that since basic conditions were being used then β-elimination of the esterified residues might occur.

2-Hydroxyethyl alginate (50mg) was shaken for 1 hour in dry methanol (20ml) with 2,2-dimethoxypropane (2ml). After the addition of sodium methoxide (10mg sodium in 10ml dry methanol), the mixture was shaken for 72 hours. The solid material was thoroughly dispersed after several hours and the solution became progressively more yellow. After neutralisation with solid carbon dioxide, the insoluble material was filtered off. The solution was evaporated (fraction A) and examined by paper chromatography (solvent 1, spray 1). A series of spots near the base line was observed and also a red-brown spot with an $R_{mannose}$ value slightly greater than 0.5. Fraction A showed an ultra-violet maximum at 277mμ.

Trimethylsilylation followed by treatment with lithium aluminium hydride and hydrolysis showed about equal portions of neutral sugars as uronic acids. It would seem likely that the incomplete reduction was due to a certain amount of ester hydrolysis having taken place during

the treatment with sodium methoxide (see experiment 17).

In a separate experiment, 2-hydroxyethyl alginate (50mg) was treated with dry methanol (100ml) containing 2,2-dimethoxypropane (10ml) for 1 hour, and then dry methanol (50ml) in which sodium (50mg) had been predissolved was added. After shaking for 24 hours, the solution was neutralised with solid carbon dioxide and then centrifuged. The supernatant solution (fraction B) was evaporated to dryness. The residue was re-treated with sodium methoxide, exactly as before. Fraction C was isolated from the supernatant solution. The residue (fraction D) was retained.

Samples of all four fractions were hydrolysed and examined by paper chromatography (solvent 3, spray 1), when spots corresponding to both mannuronic acid and guluronic acid were obtained from all four hydrolysates. Direct paper chromatography gave spots corresponding to large concentrations of oligosaccharides in each case.

Fractions B and C showed ultra-violet absorption maxima at both 237mμ and 277mμ. Fraction D showed a strong maximum at 237mμ with a weak absorption at 277mμ. The appearance of the absorption at 237mμ is characteristic of an α,β-unsaturated ester and it would seem that the 277mμ absorbing material is produced by a further elimination in the α,β-unsaturated ester.

Fractions B and D both gave a strong thiobarbituric acid reaction (resulting in an absorption maximum at 547mμ) (69), indicating the presence of 4,5-unsaturated uronic acids or esters.

Experiment 13. Isolation of oxalic acid after ozonolysis of the β-elimination products.

2-Hydroxypropyl alginate (from Manucol; 1.0g) was shaken overnight with anhydrous methanol (170ml) and 2,2-dimethoxypropane (8ml).

Methanol (170ml) and 2,2-dimethoxypropane (8ml) were shaken separately overnight, then sodium (0.34g) was dissolved in the mixture which was shaken for 1 hour. The contents of the two flasks were then mixed and shaken overnight. After neutralisation with solid carbon dioxide, the solution was evaporated to dryness. The residue was dissolved in sodium hydroxide solution (0.05N; 40ml) and left at room temperature overnight to bring about complete de-esterification. After neutralisation (dilute hydrochloric acid), ozone (79) was bubbled through the solution for 1 hour, in which time the original brown colouration disappeared. The solution was filtered to remove sandy material and concentrated to about 20ml. Hydrolysis was effected by heating on a boiling water bath for 1 hour, then calcium acetate (0.5M) was added slowly to precipitate calcium oxalate and other materials, presumably including calcium alginate. When no further precipitate appeared, the precipitate was filtered off and dissolved in sulphuric acid (1.0N), and the solution was extracted with ether (4 x 50ml). Evaporation of the ether extract gave crystals which were redissolved, treated with charcoal and recrystallised from anhydrous ether to give fine needle-shaped crystals. Recrystallisation from water gave oxalic acid dihydrate, which was identified by the X-ray powder diagram.

Experiment 14. The rate of β-elimination.

Four samples (10mg each) of 2-hydroxyethyl alginate were each treated with dry methanol (8ml) containing 2,2-dimethoxypropane (1ml), for 1 hour. To each was then added methanol (4ml) containing pre-dissolved sodium (4mg) i.e. a 3-fold excess of sodium methoxide. The solutions were neutralised by the addition of solid carbon dioxide after $2\frac{1}{2}$ hours (A), 6 hours (B), 20 hours (C) and 5 days (D). After evaporation to dryness they were dissolved in water (2-3ml). The intensity of the

yellow colouration of the solution was greatest for those samples that had been treated longest with sodium methoxide. The intensity of the absorption at 277mμ increased steadily through (A) to (D), while the intensity of the absorption at 237mμ diminished (there was no maximum at this wavelength for (D)). It would appear that the compound(s) with λ_{max} at 277mμ is being steadily formed from the compound(s) with λ_{max} at 237mμ. Paper chromatography (solvents 4 and 3) gave the following results.

Spray 4 revealed a number of spots on chromatograms run in both solvents. In solvent 4, a fast-moving spot that increased in intensity from (A) to (D) was observed, and also spots on the base-line that decreased in intensity from (A) to (D). There were also spots with intermediate R_F values but these were weaker in intensity. In solvent 3, essentially the same pattern was observed, except that the fast-moving spot was resolved into three components. With spray 1, the pattern was similar to that with spray 4. With spray 3, the pattern was again similar except that ethylene glycol (liberated by transesterification and/or de-esterification) was detected in addition to the other products. The spots gave a yellow colour soon after spraying, but after one day they turned violet-purple.

Experiment 15. Comparison of the β-elimination of mannuronic acid and guluronic acid-rich samples of alginic acid.

Samples (200mg) of the 2-hydroxyethyl esters of commercial alginic acids rich in mannuronic acid units (Manucol) and guluronic acid units (L cloustonii) were treated with methanol (80ml) containing 2,2-dimethoxypropane (5ml) for 1 hour. Sodium (90mg) predissolved in methanol (80ml) was added to each, and the samples were shaken for 1 hour (A), 4 hours (B), 14 hours (C) and 1 week (D) after which time they were neutralised with solid carbon dioxide. After evaporation to dryness, each residue was

dissolved in water and passed through a column of IR-120 resin (H^+ form). The products were isolated by freeze-drying. The ester of the mannuronic acid-rich sample was observed to disperse much quicker during the reaction than the ester of the guluronic acid-rich sample. Samples (C) and (D) from the mannuronic acid-rich alginic acid gave syrups on freeze-drying, perhaps because they were of such low molecular weight that the freezing point of the solution was depressed too far for it to remain frozen.

Ultra-violet spectra showed that the absorption at 235mμ was very weak for all the guluronic acid-rich samples; after prolonged treatment the absorption at 277mμ was observed, however. In contrast, the mannuronic acid-rich samples showed a very pronounced absorption maximum at 235mμ after 1 hour, which diminished steadily with time until it had almost disappeared after 14 hours and 1 week; at the same time, the absorption at 277mμ, which was not detectable after 1 hour, steadily increased until it was very pronounced after 14 hours and 1 week. It was also observed that an absorption at 240mμ appeared in both the samples which had been treated for 1 week. This was most pronounced in the mannuronic acid-rich sample. The degrees of polymerisation (D.P.) of the products were estimated from data given by Macmillan, Pfaff and Vaughn(77). The value found by these workers for the extinction coefficient of 4,5-unsaturated oligouronic acids obtained from pectic acid, was assumed to hold for the products in the present experiment. Samples were dissolved in acetate buffer (0.1M), pH 3.7, to give solutions that were approximately 1.8×10^{-4}M (assuming a D.P. of 4). In acetate buffer the only ultra-violet absorption observed for all the samples was the 235mμ absorption i.e. no 277mμ absorption was obtained. This was perhaps due to a tautomeric shift of the 277mμ

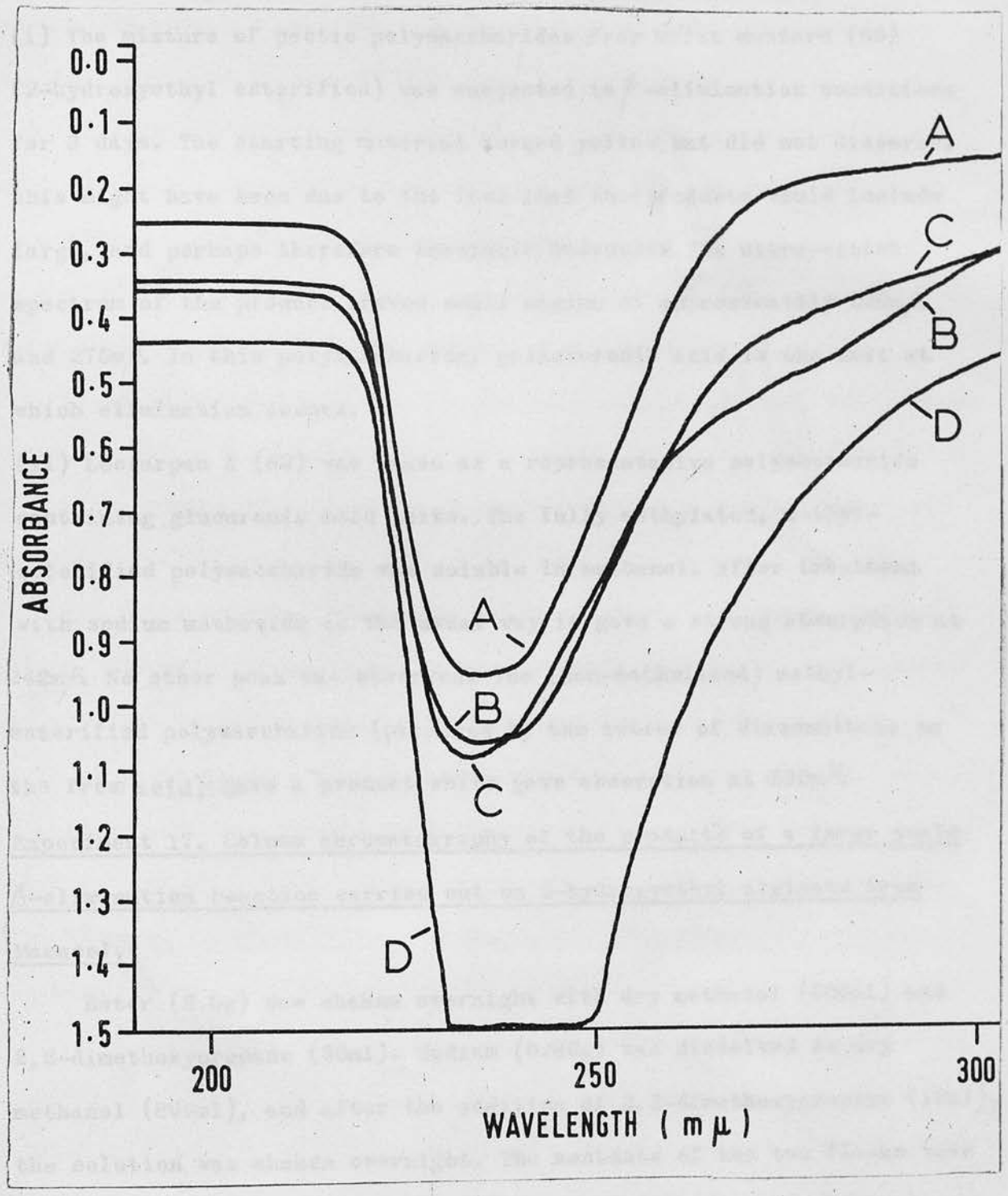
absorbing material to the lower wavelength (see discussion). The D.P. of the various samples was based on the absorbance at 235mμ. The spectra obtained for the mannuronic acid-rich sample are reproduced in figure 8. The following results were obtained

Table 4.

Time of reaction	Approximate D.P. of product from:	
	Manucol alginate	L.cloustonii alginate
1 hour	8	23
4 hours	7	13
14 hours	7	11
7 days	1-2	9

The Manucol ester was also subjected to β-elimination with varying concentrations of sodium methoxide. Concentrations that were 5-fold, 10-fold and 25-fold greater than in the experiment above were used for a reaction period of 1 hour. The D.P. of the products did not appear to be any lower than before; the only difference that was observed at the higher methoxide concentrations was that the rate of formation of the material absorbing at 277mμ was increased.

2-Acetoxyethyl di-O-acetylalginate (from Manucol) was also subjected to β-elimination; it seemed possible that this might overcome any tendency for the reaction to be arrested by the presence of crystalline regions in the solid phase. The D.P. of the product after reaction for 1 hour was approximately 4. Since the acetylated material was free from contaminants whereas the 2-hydroxyethyl ester was only approximately 70% pure (see experiment 9(c)) this would suggest that elimination in the acetate is proceeding about three times faster than in the ester itself.



Ultra-violet spectra of the products of β-elimination from Manucol alginate.

Figure 8.

Experiment 16. β-Elimination of other polysaccharide esters.

(i) The mixture of pectic polysaccharides from white mustard (60) (2-hydroxyethyl esterified) was subjected to β-elimination conditions for 3 days. The starting material turned yellow but did not disperse; this might have been due to the fact that the products would include large, and perhaps therefore insoluble molecules. The ultra-violet spectrum of the product showed small maxima at approximately 240mμ and 275mμ. In this polysaccharide, galacturonic acid is the unit at which elimination occurs.

(ii) Leocarpan A (80) was taken as a representative polysaccharide containing glucuronic acid units. The fully methylated, methyl-esterified polysaccharide was soluble in methanol. After treatment with sodium methoxide in the usual way it gave a strong absorption at 242mμ. No other peak was observed. The (non-methylated) methyl-esterified polysaccharide (prepared by the action of diazomethane on the free acid) gave a product which gave absorption at 230mμ.

Experiment 17. Column chromatography of the products of a large scale β-elimination reaction carried out on 2-hydroxyethyl alginate from Manucol.

Ester (2.0g) was shaken overnight with dry methanol (800ml) and 2,2-dimethoxypropane (30ml). Sodium (0.80g) was dissolved in dry methanol (800ml), and after the addition of 2,2-dimethoxypropane (10ml), the solution was shaken overnight. The contents of the two flasks were then mixed and shaken for 1 hour. This period of time was chosen to minimise the production of the 277mμ absorbing material, which would have complicated the mixture of products. After neutralisation with solid carbon dioxide the solution was evaporated to dryness and the residue was dissolved in water. The solution was filtered to remove

some insoluble material and then was passed through an ion exchange column (IR-120, H^+). The complete removal of sodium ions was confirmed by the flame test on the effluent.

A cellulose column was prepared and equilibrated with butanol : ethanol : water (2 : 2 : 1) solvent. The decationised solution was evaporated to a small volume, and then butanol and ethanol were added to give the final proportions of 2 : 2 : 1. Precipitation of some of the dissolved material occured at this stage and this was removed by filtration, suspended in water (it was only partly soluble), and freeze-dried (0.480g). The degree of polymerisation, calculated from the ultra-violet absorption of a solution in acetate buffer (see experiment 15) was 13.

The brown solution containing soluble material was loaded on the column. Elution with butanol : ethanol : water (2 : 2 : 1) was at 300ml per day, and fractions were collected automatically (25ml). On the basis of their ultra-violet spectra, the fractions were combined together as follows and concentrated to dryness.

Table 5.

Tube Numbers	Appearance	Solubility properties	Weight
1-19	oil	Insoluble in methanol, soluble in chloroform.	less than 10mg.
20-21	oil	Soluble in methanol	15mg.
22-23	oil	Soluble in methanol	76mg.
24	oil	Soluble in methanol	80mg.
25-29	oil	Soluble in methanol	160mg.
30 onwards	oil at first, later becomimg amorphous solid.	Soluble in methanol	215mg.

Tubes 1-19: This gave a sharp λ_{max} at about 235mμ. Tube 8 contained the greatest concentration of this material, and tubes 1 and 19 the smallest (as judged by ultra-violet spectra). The combined fraction was contaminated with smaller amounts of cellulose and silicone oil. Because of this, and also because of the small weight, it was not examined further.

Tubes 20 and 21: On paper chromatograms this gave a fast-moving spot which showed very distinctive behaviour towards spray 1. The nature of this material is discussed in more detail in experiment 18.

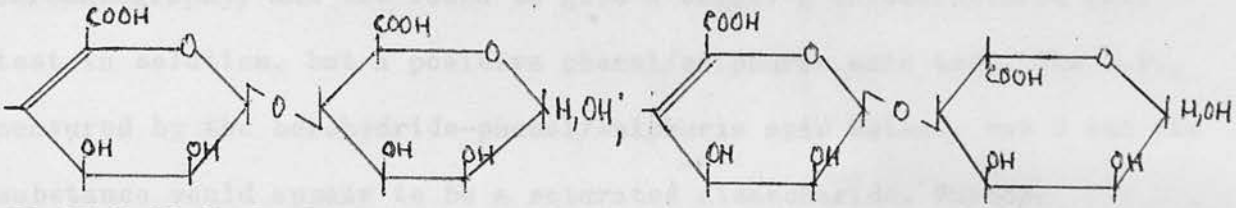
Tubes 22 and 23: Paper chromatography showed that the major component was the same as in tubes 20 and 21, although there was some tailing.

Tube 24: This again contained the fast-moving material, followed by "trailing", followed by a spot which corresponded to the material obtained almost pure in tubes 25-29

Tubes 25-29: Paper chromatography (solvent 4, spray 1) showed a single red-brown spot, with some trailing material which moved a little faster than glucuronic acid, but with considerable trailing. The fact that the mixture gave a positive ester test, (81), suggested that the trailing might be due to some esterified material. This seemed to be confirmed by the fact that after standing in 0.05N sodium hydroxide overnight at room temperature, only slight trailing was observed in the electrophoretogram. The apparent degree of polymerisation (D.P.) as determined by a potassium borohydride- phenol/sulphuric acid modification of Peat, Whelan and Roberts' method (82) was 1.94. After hydrolysis and paper chromatography, both mannuronic acid and guluronic acid were observed. The ultra-violet spectrum showed a maximum at 235mμ, suggesting the presence of 4,5-unsaturated uronic acid units. The infra-red spectrum showed the absorptions characteristic

of α,β-unsaturated acids at 1640-1650cm^{-1} and 1720cm^{-1}. The accumulated evidence suggested that the fraction contained a mixture of

Figure 9.



and some of their esterified derivatives.

Tubes 30 onwards: This showed the presence of the component which was the major one in tubes 25-29, together with material that trailed on paper chromatograms (solvent 4, sprays 1 and 4) right back to the base line. Trailing was also observed on paper electrophoretograms, but this disappeared after treatment of the mixture with 0.05N sodium hydroxide at room temperature overnight. The mobility of the spot then observed was similar to the corresponding one from tubes 25-29. The average D.P. of the material in this fraction was 4.5. The ultra-violet spectrum showed a maximum at about 235mμ and it would therefore appear likely that this fraction contained a mixture of oligouronic acids with 4,5-unsaturated terminal non-reducing units and with a proportion of the units being esterified.

Further examination of the unsaturated oligosaccharides was carried out, giving the following results. The fractions obtained from tubes 25-29 and from tubes 30 onwards were separately treated with 0.05N sodium hydroxide at room temperature overnight in order to effect de-esterification and to simplify the mixtures. After neutralisation with IR-120 (H^+) resin, the solutions were concentrated and examined by paper chromatography (solvent 5). The product from tubes 25-29 showed two fast-moving spots and a slower moving one. These all gave a positive reaction with spray 1 and a negative one with spray 4. One of the

faster spots corresponded closely with mannuronic acid in R_F value. The slow spot was more intense in the product from tubes 30 onwards. Some of the material in this zone was purified by thick paper chromatography, and was found to give a negative thiobarbituric acid test in solution, but a positive phenol/sulphuric acid test. The D.P., measured by the borohydride-phenol/sulphuric acid method, was 2 and the substance would appear to be a saturated disaccharide. Further confirmation of this was that it moved at the same rate as the disaccharide from hydrolysed alginic acid. This would seem to indicate that the unsaturated end-group had been removed by hydrolysis probably by the formic acid in the solvent system.

In addition to the saturated disaccharide the de-esterified products from tubes 30 onwards showed traces of the two faster-moving spots that were observed in the contents of the de-esterified products from tubes 25-29, and also a series of spots that were slower-moving than the disaccharide. Thiobarbituric acid-positive material was observed only in the region of the origin for both fractions.

Experiment 18. Further examination of the main component in tubes 20 and 21.

The main component gave a fast spot on chromatograms run in solvent 4, which gave an immediate yellow colour with p-anisidine hydrochloride spray, even in the cold. The spot changed to a pink colour on standing overnight. After elution of the material from a paper chromatogram, the D.P. was found to be unity (borohydride-phenol/sulphuric acid method) and the ultra-violet spectrum showed a maximum at 245mμ (see below, however). With the thiobarbituric acid spray there was no strong colouration in the region of the spot that could be detected with p-anisidine hydrochloride, and the substance would therefore appear not to be an α,β-unsaturated ester. The possibility that it might have been

an α-keto ester (17) was investigated using ethyl pyruvate as a reference compound, but the test (83) was negative. Ethyl pyruvate had an ultra-violet maximum at 220mμ - significantly different from the unknown which had a maximum at 245mμ. The infra-red spectrum (taken by spreading the oil on sodium chloride plates) showed two carbonyl peaks, and no olefinic peak.

From the evidence listed above it is clear that the unknown was probably a decomposition product of a 4,5-unsaturated uronic acid unit. The possibility that it might be a β-diketone was tested with ferric chloride, acetylacetone being used as a control. A negative reaction was given by the unknown. On the other hand an ester test was positive, and the unknown was shown to be non-ionic by electrophoresis. Attempted de-esterification by treatment with 0.05N sodium hydroxide at room temperature overnight, then neutralisation (IR-120 resin, H^+ form), seemed to result in destruction of the material because no product could be detected by electrophoresis using the p-anisidine hydrochloride spray.

The chromophore responsible for the ultra-violet maximum at 245mμ was eventually shown to be present in an impurity, and not in the main component of this fraction. The absorption was destroyed by ozonolysis, indicating the presence of an olefinic double bond. There was also evidence that the product with the ultra-violet maximum at 245mμ might be derived from a 4,5-unsaturated uronic acid by the action of acid. When some material from tubes 30 onwards was left standing in 0.1N sulphuric acid for periods up to 20 hours, there was no change in the ultra-violet spectrum. When the acid solution was warmed to 80° for 1 hour, however, the maximum shifted from 235mμ to 255mμ. After neutralisation with barium carbonate and filtration, the maximum

shifted again, to 245mμ i.e. to the maximum of the unknown. There was a resemblance to the unknown not only in the position of the maximum, but also in the effect of pH. When the material from tubes 22 and 23 was acidified, the absorption shifted from 244mμ to 253mμ; subsequent addition of excess alkali shifted the absorption back to 244mμ. The material with the absorption at 244mμ might therefore have arisen from the unsaturated uronic acid units during the treatment with IR-120 (H^+) in the work-up.

The non-identity of the ultra-violet absorbing substance with the substance giving the distinctive yellow turning pink colouration with p-anisidine hydrochloride, was established by the elution of strips cut from a chromatogram run in solvent 4. The strip which gave the maximum ultra-violet absorption did not coincide with the central zone of the p-anisidine positive spot, but corresponded to an appreciably lower R_F. This experiment was performed with part of the contents of tubes 22 and 23, and with the acid-treated material from tubes 30 onwards, with essentially the same results in both cases. The ultra-violet absorbing component was in fact located more conveniently by viewing under an ultra-violet lamp (350mμ). Further evidence pointing to the non-identity of the two substances was that ozonolysis, which destroyed the ultra-violet absorption, had no effect on the p-anisidine hydrochloride-positive material. The p-anisidine-positive material could also be located with the periodate-benzidine spray, indicating the presence of contiguous hydroxyl groups in this compound, but the ultra-violet absorbing substance could not be located in this way.

The possible interconvertibility of the ultra-violet absorbing and p-anisidine hydrochloride-positive substances was next examined.

When the product from tubes 22 and 23 was heated at 80° with 1N acid for 1 hour, the ultra-violet absorption remained unaltered. It would therefore seem that the two components are not interconverted in acid. Moreover, when the neutralised acid-treated product from tubes 30 onwards was subjected to electrophoresis, there was no evidence for the formation of neutral material with the distinctive yellow turning pink colouration. Some neutral material was formed, but this did not react with p-anisidine hydrochloride in the cold, and it gave a reddish-brown colour on heating. This was presumably a mixture of lactones formed after partial hydrolysis of the oligosaccharides. It follows that the p-anisidine-positive material is probably not an artefact from the resin treatment of the unsaturated products of β-elimination. Later experiments (experiment 22) indicated that in fact the ultra-violet absorbing material was 2-furoic acid.

Attention was turned next to the nature of the p-anisidine hydrochloride-positive unknown. It appeared likely that this substance was a breakdown product formed by the action of base on α,β-unsaturated uronic acid units, but a number of possible breakdown paths were eliminated by the evidence available on the nature of the substance. A possibility that was not inconsistent with the evidence is breakdown by a decarbonylation reaction as shown in figure 10. If the p-anisidine positive material had the structure (I), then it should give 2-deoxyribitol on reduction. This is not a complete characterisation of the structure, because 2-deoxyribitol could also arise from the products shown in figure 11, which could also arise by base-catalysed fragmentation reactions of the α,β-unsaturated acid or its tautomer.

No precedent has been found in the literature for the decarbonylation reaction proposed although such may exist and it seems plausible

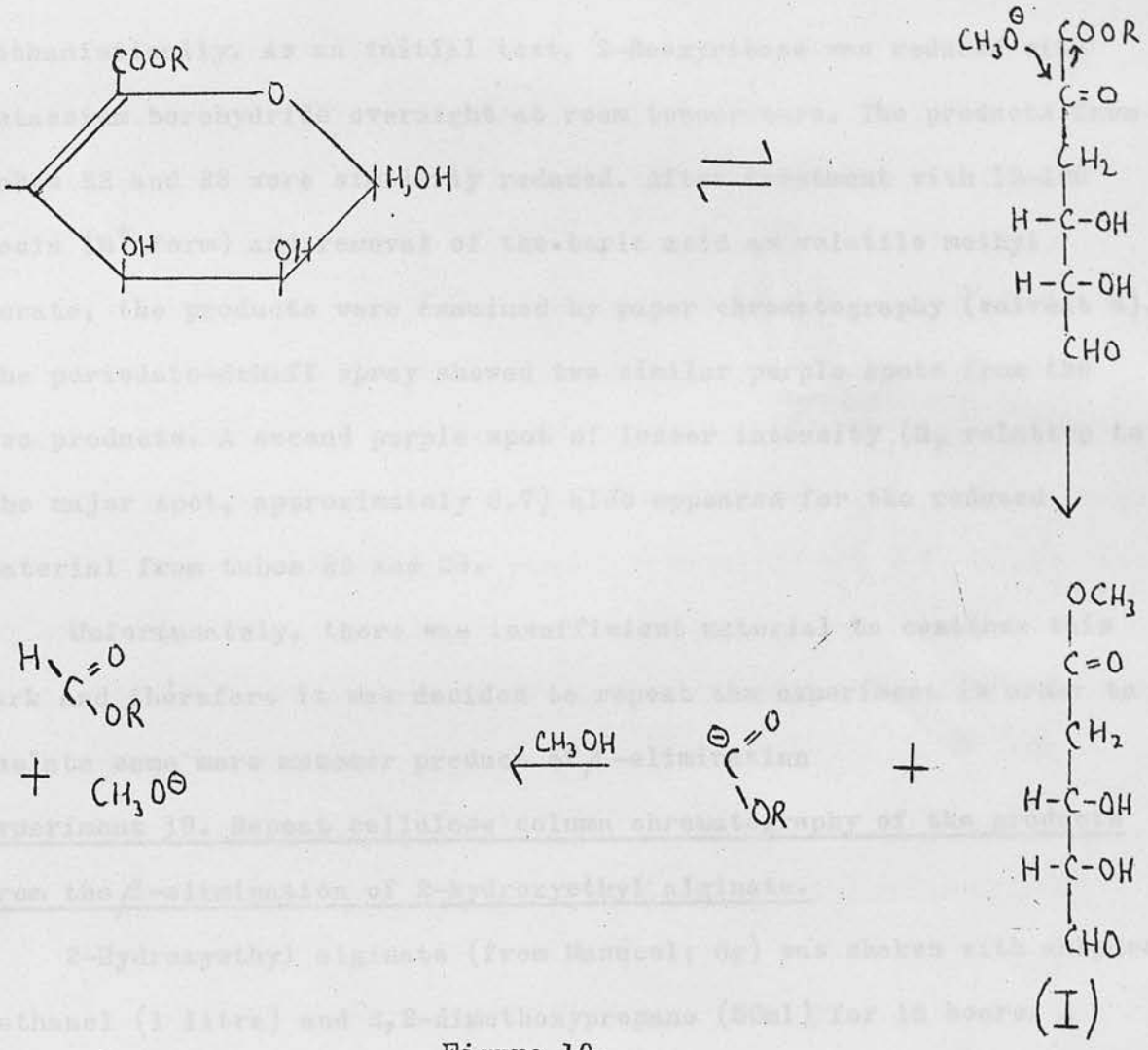


Figure 10.

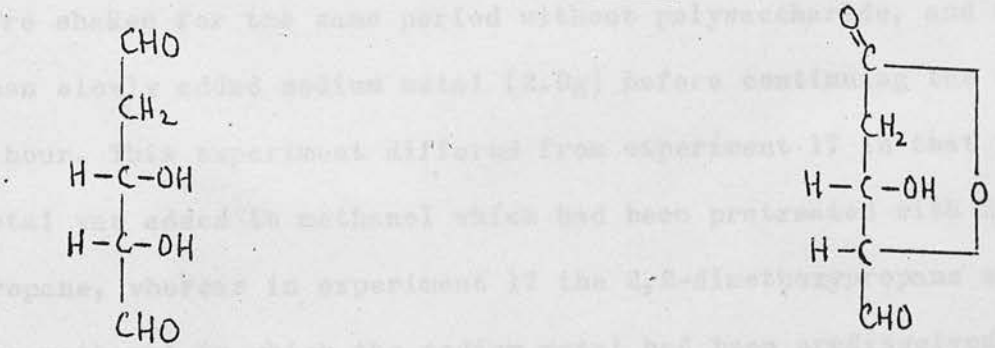


Figure 11.

mechanistically. As an initial test, 2-deoxyribose was reduced with potassium borohydride overnight at room temperature. The products from tubes 22 and 23 were similarly reduced. After treatment with IR-120 resin (H^+ form) and removal of the boric acid as volatile methyl borate, the products were examined by paper chromatography (solvent 4). The periodate-Schiff spray showed two similar purple spots from the two products. A second purple spot of lesser intensity (R_F relative to the major spot, approximately 0.7) also appeared for the reduced material from tubes 22 and 23.

Unfortunately, there was insufficient material to continue this work and therefore it was decided to repeat the experiment in order to isolate some more monomer product of β-elimination

Experiment 19. Repeat cellulose column chromatography of the products from the β-elimination of 2-hydroxyethyl alginate.

2-Hydroxyethyl alginate (from Manucol; 6g) was shaken with anhydrous methanol (1 litre) and 2,2-dimethoxypropane (50ml) for 16 hours. A further quantity of methanol (1 litre) and 2,2-dimethoxypropane (50ml) were shaken for the same period without polysaccharide, and to this was then slowly added sodium metal (2.0g) before continuing the shaking for 1 hour. This experiment differed from experiment 17 in that the sodium metal was added to methanol which had been pretreated with 2,2-dimethoxypropane, whereas in experiment 17 the 2,2-dimethoxypropane was added to the methanol in which the sodium metal had been predissolved.

The contents of the two flasks were then mixed together and shaken for 1 hour, then neutralised by addition of solid carbon dioxide. After evaporation to a small volume, water was added and then evaporation was continued to remove all the methanol. The solution was filtered to remove some gritty sandy material (see experiment 9(c)) and then treated

with IR-120 resin (H^+ form). After being concentrated further, the solution was mixed with butanol and ethanol to give the final proportions, butanol : ethanol : water (2 : 2 : 1). The precipitate which formed was removed by filtration and dried in a vacuum oven (weight: 2.74g). The solution was evaporated to dryness and re-dissolved in butanol : ethanol : water (2 : 2 : 1). A small part remained insoluble - a light brown translucent gum (0.150g).

The dark brown solution was loaded onto a cellulose column, which was then eluted with butanol : ethanol : water (2 : 2 : 1) at the rate of 450ml/day. Fractions (30ml) were collected, pooled as shown in the following table and evaporated to dryness in weighed flasks which were reweighed after drying.

Table 6.

Tube Numbers	Weight of combined contents (mg)
1	10
2	85
3	100
4	445
5	420
6-7	295
8-11	126
12-21	188
22-70	167
71onwards	87

The contents of tubes 1 and 2, when examined by paper chromatography in solvent 4, showed component(s) which trailed from just behind the solvent front and which were visible without spraying, under the ultra-violet lamp, and gave positive reactions with p-anisidine hydrochloride

and thiobarbituric acid sprays. The same pattern emerged when these fractions were examined by paper chromatography in solvents 1 and 2. In solvent 6, and using the thiobarbituric acid spray, fraction 2 was resolved into three distinct spots having R_F values of 0.9, 0.5 and 0.25, in the approximate relative concentrations 70%, 20% and 10% respectively (judged from spot intensities). Electrophoresis of fraction 2 showed 60–70% neutral material and 30–40% acidic material. After de-esterification by treatment with 0.05N sodium hydroxide at room temperature overnight, followed by neutralisation (IR-120 resin, H^+ form), re-examination by paper chromatography (solvent 6) showed no significant change. The difference between the several components of fraction 2 (and probably also fraction 1) was therefore not simply in degree of esterification. It seems likely the di-, tri- and tetra-saccharide derivatives were present.

Solvent 7 was another good solvent system for the paper chromatography of de-esterified fractions.

Despite exhaustive examination of the early fractions from the column, there was no evidence for the presence of the fast-moving component which was discussed at length in experiment 18 and which gave a characteristic yellow turning pink spot on paper chromatograms sprayed with p-anisidine hydrochloride.

It had been hoped to isolate this substance and prove its structure. The only difference between this experiment and the previous experiment when this substance was isolated has been mentioned in the first paragraph of this section and it is not easy to see how the reaction products would be affected by this difference, except that the reaction would be halted earlier because the base would be consumed earlier in the absence of pre-drying.

A second puzzling result was the apparent absence of the α-keto acid or α-keto ester which would be expected by ketonisation of the 4,5-unsaturated monomer. The α-keto-ester test, using ethyl pyruvate as reference compound, was negative for fraction 1, 2 and 3. This test was also negative for the whole mixture of products, after β-elimination had been allowed to proceed for 3 days.

Experiment 20. Attempted preparation of the α-keto-ester monomer by mild hydrolysis of the 4,5-unsaturated products of β-elimination.

An attempt was made to prepare the α-keto ester monomer from the mixture of unsaturated oligosaccharides by acid hydrolysis that was sufficiently mild to avoid decomposition. It has been previously shown that the monomer exists in the keto rather than the enol form (17), and therefore the progress of this hydrolysis may be followed by the disappearance of the absorption at 235mμ. The mixture of products from β-elimination was treated with sulphuric acid of various concentrations at 80°, and the ultra-violet spectrum was recorded at intervals. In 1.0N acid, it was found that the 235mμ band disappeared after 45 minutes at 80°, and that after 2 hours a new band (presumably a degradation product) began to appear at 255mμ (compare experiment 18). With 0.1N acid, the respective times were 1 hour and overnight. In an attempt to detect the α-keto ester, the sample was therefore treated with 0.1N sulphuric acid at 80° for 2 hours and then neutralised (calcium carbonate) and tested with the reagents for the α-keto ester test. The result was negative. Similar tests were carried out after time intervals of 0, 10, 20 and 30 minutes, but in each case the result was negative. Similar experiments with ethyl pyruvate showed that this ester survived acid treatment for periods up to at least 3 hours. It would seem (not unexpectedly) that the sugar α-keto ester is more labile than ethyl pyruvate.

Experiment 21. Proof that the unsaturated end-units are esterified in the β-elimination products.

One of the reasons for this investigation was that if the unsaturated end-groups were present as the carboxylate anions (figure 12), then these might be poor leaving groups in a further elimination to give the products shown in figure 13.

Figure 12.

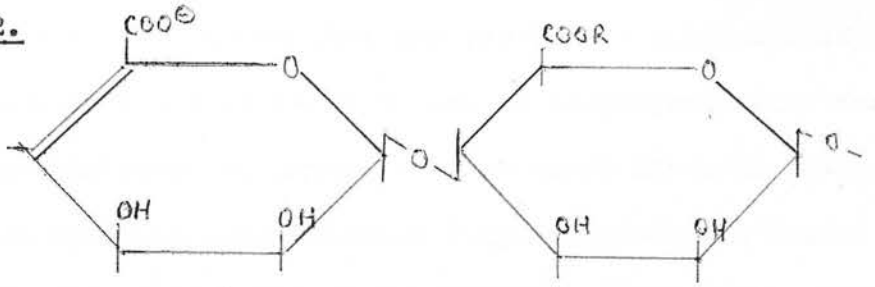
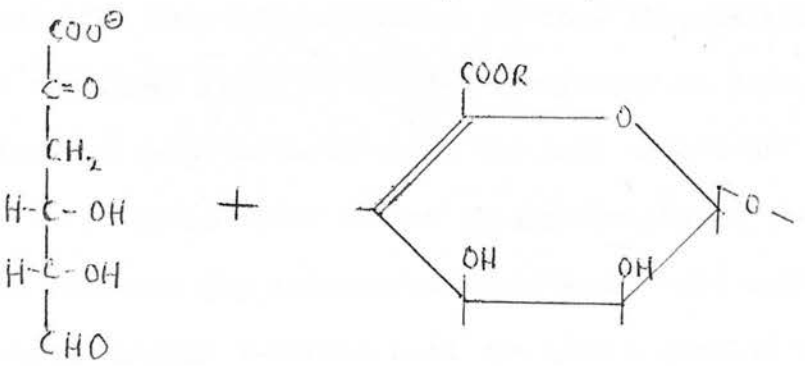


Figure 13.



This might account for both the failure to detect the α-keto acid monomer in the β-elimination products, and the fact that the β-elimination reaction does not go to completion before it stops.

The experimental proof was very simple. Fraction 3 from the column (experiment 19) was dissolved in water and left with potassium borohydride overnight. The absorption at 235mμ disappeared completely from the ultra-violet spectrum. The unsaturated end-group was therefore esterified because the salt would not have been reduced (84).

Experiment 22. Proof that 2-furoic acid is not the monomer product of the β-elimination reaction.

The recent report (85) that 2-furoic acid is an acid degradation product of the 4,5-unsaturated oligosaccharides derived from alginic acid by enzyme action, prompted the investigation of whether 2-furoic acid might occur in the products of the action of sodium methoxide on alginate esters. It seems that the previously unidentified product of mild acid hydrolysis of the unsaturated oligosaccharides formed by methoxide-catalysed β-elimination, and which shows an absorption maximum at 255mμ in acid solution (experiment 20) is indeed 2-furoic acid. The evidence for this identification is that the absorption maximum of this substance could be shifted reversibly to 245mμ in alkaline solution and 2-furoic acid shows the same behaviour. The substance was also isolated after column chromatography of the products of β-elimination (without any intentional acid treatment) and this raised the question whether 2-furoic acid was also a product of base-catalysed degradation of the 4,5-unsaturated oligosaccharides. If so, its likely origin would be via a Cannizaro reaction from furfuraldehyde, itself derived by cyclisation and decarboxylation of an unsaturated uronic acid. Indeed, β-elimination products had previously been observed to give an absorption maximum in aqueous solution at 277mμ (experiment 12), which corresponds to the maximum for furfuraldehyde. The isolation of furfuraldehyde and 2-furoic acid was therefore attempted in the following experiment.

2-Hydroxyethyl alginate (MCI; 0.40g) was shaken with methanol (150ml) and 2,2-dimethoxypropane (10ml) for 16 hours. Sodium methoxide (150ml; 0.1%; containing 5% dimethoxypropane) was then added and the solution was shaken for 6 days prior to neutralisation with solid

carbon dioxide. At this stage the supernatant solution gave an absorption maximum at 240mμ. An aqueous solution of the entire products (prepared after evaporation of the methanol) showed a maximum at 279mμ with a small inflection at 230mμ. To this aqueous solution was added sodium bicarbonate. Extraction with ether then removed only material with maxima at 235mμ and (smaller) 262mμ. The spectrum of the aqueous solution was essentially unchanged by ether extraction. After acidification, the extraction with ether was repeated. The ether extract gave a similar spectrum to the previous ether extract. The acidic aqueous solution showed a maximum at 235mμ only. Had furfuraldehyde or 2-furoic acid been present, they would have been extracted with ether before or after acidification respectively.

Further confirmation was obtained that the elimination product with an absorption maximum at 279mμ was not furfuraldehyde, as follows. Furfuraldehyde (authentic material) was left in alkaline solution (33% aqueous sodium hydroxide) overnight, when the absorption maximum shifted as expected to 246mμ as a result of the Cannizaro reaction by which it was converted to 2-furoic acid and furfuryl alcohol. In contrast the β-elimination product with the maximum at 279mμ was found to be unaltered by this treatment.

The identities of the substances which show the maxima at 240mμ and 279mμ are unknown. It has been shown, however, that the substance with the 279mμ absorption is formed from the 240mμ absorbing material by simply evaporating the methanol solution to dryness and dissolving in water. The change is not reversible, because evaporation of the aqueous solution and dissolution of the residue in methanol does not cause the disappearance of the 279mμ absorption. Both the absorption at 240mμ and at 279mμ showed reversible shifts with pH. When the 240mμ

absorbing material was made acidic the maximum shifted to 245mμ, and it moved back again to appear as an inflection at 240mμ when the solution was re-made alkaline. Alkaline treatment alone left the absorption unaffected. The 279mμ maximum was also unaffected by alkali, but when the solution was slowly made acidic the absorption at 279mμ disappeared slowly and an inflection appeared at 232mμ. The original spectrum re-appeared when the solution was then made alkaline.

It seems probable that the substance responsible for the 279mμ absorption is an ester of some sort, because the peak disappears gradually in the presence of potassium borohydride. In contrast, if the solution is made alkaline (to 0.1N sodium hydroxide) and left overnight before addition of the borohydride, the spectrum is subsequently unaffected by the reducing agent.

Experiment 23. Elimination studies on model compounds.

Since a successful identification of the monomer product(s) from the β-elimination reaction on alginate esters had not been forthcoming, attention was turned to studies on monosaccharide model compounds.

23(a). Tetrahydropyranyl-2,3,4-tri-O-tetrahydropyranyl-α/β-glucopyranoside uronic acid tetrahydropyranyl ester as a model compound.

Glucuronic acid (20mg) was shaken for 16 hours with dihydropyran (2ml), tetrahydrofuran (2ml) and concentrated hydrochloric acid (2 drops) (86). The glucuronic acid dissolved slowly as the reaction proceeded, and thin layer chromatography indicated the formation of a non-polar substance that was presumably the above-named derivative. Sodium bicarbonate was added to neutralise the solution. After shaking for a further 16 hours, 2,2-dimethoxypropane (0.2ml) was added followed by further shaking for 2 hours. Sodium methoxide (0.1%, containing 5% dimethoxypropane) was added (4ml), and after 10 minutes

at room temperature the ultra-violet spectrum was recorded. The only absorption was at 247mμ with no absorption at 235mμ. It would seem that this compound, as with the polysaccharide, the initial formation of a 4,5-unsaturated product is followed by further reactions.

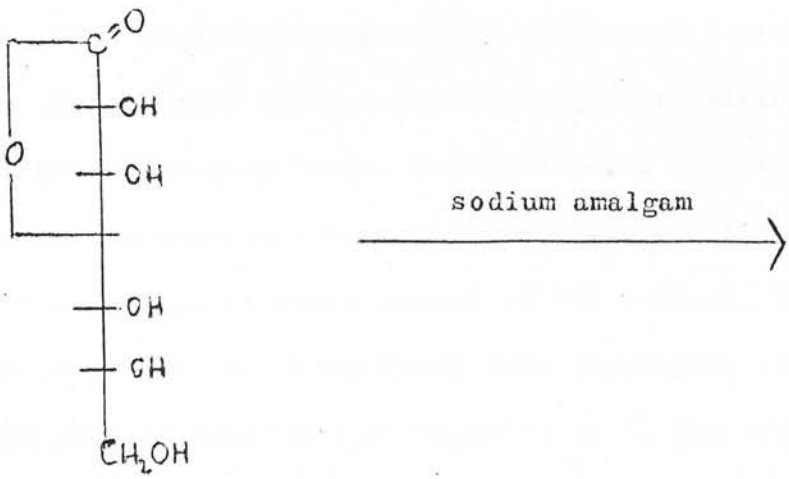
23(b). Methyl-2,3,4-tri-O-methyl-β-glucopyranoside uronic acid methyl ester as a model compound.

Methanol (50ml), 2,2-dimethoxypropane (4ml) and the above named compound (200mg) were shaken at room temperature for 16 hours. Sodium methoxide (10ml; 0.1%; containing 5% dimethoxypropane) was added and the mixture was shaken for 16 hours. After neutralisation with carbon dioxide, several drops of the mixture were removed and added to water and this solution was used to record the ultra-violet spectrum. A maximum at 239mμ was observed. When the solution was acidified the maximum shifted to 252mμ. On making the acidic solution alkaline there was no further change. Alkali also had no effect on the absorption of the original solution. It would seem that the acidification had caused some chemical change rather than a shift to a tautomer.

The neutralised reaction mixture was evaporated to dryness to give a residue which was insoluble in water; the ultra-violet spectrum was therefore recorded in tetrahydrofuran. A maximum was observed at 245mμ. The absence of olefinic protons in the n.m.r. spectrum (in deutero-chloroform) was probably due to very incomplete reaction, because the infra-red spectrum in chloroform was very similar to the starting material.

Experiment 24. Syntheses of methyl β-D-gulopyranosiduronic acid.

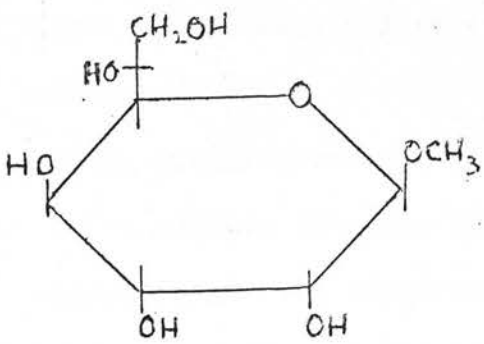
As an aid to elimination studies on model compounds the synthesis of methyl β-D-gulopyranosiduronic acid was attempted. The sequence of reactions used is shown in figure 14.

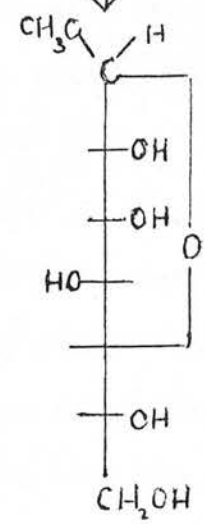


D-glycero-D-gulo-heptono-1,4-lactone

D-glycero-β-D-gulo-heptose

methanolic hydrogen chloride

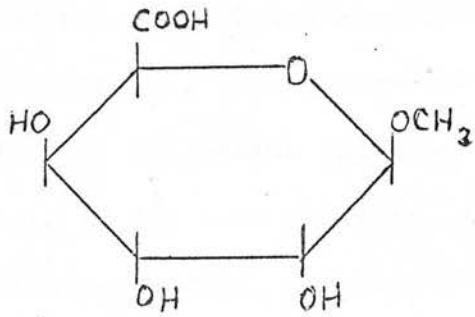




(i) Periodic acid

(ii) Bromine

methyl-β-D-glycero-D-gulohaptopyranoside



methyl β-D-gulopyranosiduronic acid

Figure 14.

24(a). Purification of D-glycero-D-gulo-heptono-1,4-lactone.

42g crude D-glycero-D-gulo-heptono-1,4-lactone (87)(prepared by Dr. D. A. Rees) was recrystallised by dissolving in ⅔ of its own weight of boiling water, adding a small amount of decolourising carbon, filtering through a heated Buchner funnel, transferring to a beaker and diluting with an equal volume of 95% ethanol. The solution was allowed to cool to room temperature, left overnight, stirred to prevent caking, and crystallisation was completed at 1°. The crystals were filtered with portions of 80%, 90% and absolute ethanol. The product separated in small prisms (M.Pt. 151-152°; Yield 30g). The supernatant solution of aqueous ethanol was evaporated to a small volume and diluted with an equal volume of absolute ethanol. A further 6g of pure product was obtained.

24(b). Preparation of D-glycero-β-D-gulo-heptose (87).

A mixture of water (200ml) and 20% sulphuric acid (5ml) was stirred until the temperature reached 0° or less. Then, D-glycero-D-gulo-heptono-1,4-lactone (34g) and fresh 2.5% sodium amalgam (57g) were dropped in and 20% sulphuric acid was added dropwise to keep the acidity near pH 3. When all the amalgam had reacted and with the temperature being maintained between zero and 5°, a second 57g batch was added and then a further eight 114g batches at intervals of 20-30 minutes. When the reaction was complete, the aqueous solution was decanted from the mercury and filtered. The sodium sulphate that separated was dissolved in water and added to the main solution. A few drops of phenolphthalein indicator solution were added, and then N sodium hydroxide solution was added carefully to neutralise the sulphuric acid and convert any unchanged lactone into the sodium salt of the heptonic acid; to ensure completion of the latter step, the solution showed the characteristic

red colour of phenolphthalein for 30 minutes. The solution was then neutralised with dilute sulphuric acid, filtered with a small amount of decolourising carbon and concentrated. When bumping became troublesome because of the crystallised sodium sulphate, the solid was redissolved by heating and the solution was poured into 600ml of hot 85% ethanol. The mixture was allowed to cool to room temperature and the precipitated sodium sulphate and sodium D-glycero-D-gulo-heptonate were filtered and washed three times with 160ml portions of 85% ethanol The filtrate was concentrated to a thick syrup from which crystals were obtained (16g). After recrystallisation from aqueous ethanol pure D-glycero-β-D-gulo-heptose was obtained (M.Pt. 195°; Yield 7.5g; 22%).

24(c). Preparation of methyl-β-D-glycero-D-guloheptopyranoside (88).

D-glycero-β-D-gulo-heptose (7.0g) was refluxed with 5% methanolic hydrogen chloride for 12 hours. After neutralisation with silver carbonate and decolourisation with carbon, the solution was concentrated to a thick syrup. Crystallisation of the heptoside did not take place and the components were separated on a cellulose column with butanol : ethanol : water (2 : 2 : 1) as eluting solvent. The fractions corresponding to the main component in the mixture were pooled and concentrated to a syrup from which methyl-β-D-glycero-D-gulo-hepto-pyranoside was obtained. The first two crops of crystals were collected and recrystallisation was carried out with ethanol. (M.Pt. 166-168°; Yield 1.38g; 19%). Further crystallisation was observed but this was slow in taking place.

24(d). Preparation of methyl β-D-gulopyranosiduronic acid.

Preliminary experiments indicated that the optimum length of time for the exocyclic oxidation of the heptoside was 30 minutes (as judged by electrophoresis of the products after further oxidation with

bromine). The following procedure was therefore adopted:-

Heptoside (1g) was dissolved in water (20ml) and cooled in an ice-bath. Periodic acid solution (20.5ml of 0.25M periodic acid, 1.1 mole) was added and the solution was left for 30 minutes. Warm barium hydroxide was then added until pH 9.5 to prevent further oxidation. After centrifugation, the supernatant solution was decanted and bromine was added to give a separate layer. After leaving in the dark for 48 hours, excess bromine was removed by aereation and then excess silver carbonate was added and left for 16 hours to neutralise the solution. The insoluble silver carbonate and silver bromide were filtered off and the aqueous solution was deionised with IR-120 H^+ resin. Concentration gave a thick syrup. Even after prolonged standing, crystals did not appear and therefore the product was purified on a DEAE-Sephadex A 25 column in the formate form. Neutral sugar was eluted with water and acidic products with a water-- 5% formic acid gradient over 1.5 litres. A single product travelling at the same rate as glucuronic acid on electrophoresis (sprays 2 and 5) was obtained (0.58g; 62%).
The p.m.r. spectrum in deuterium oxide solution showed the following features:

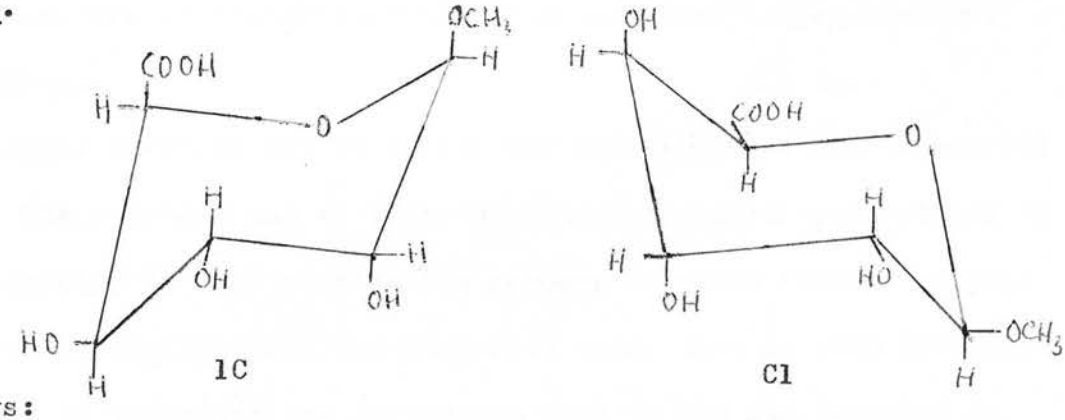
5.35τ - Complex multiplet corresponding to two protons probably a doublet (spacing 7c/s) with a singlet placed between. This is assigned to H-5 plus H-1 because H-1 usually occurs at lower field than other sugar ring protons and H-5 is presumed to be deshielded by the carboxylic acid group (A comparison of the CH_2 protons in n-propanol with the CH proton in lactic acid showed that the latter occurs at a lower field. This supports the above presumption).

5.85τ - Singlet corresponding to two protons.
6.20τ - Singlet (?) corresponding to one proton, partly merged with the singlet at 6.38
} Assigned to H-2 plus H-3 plus H-4

6.38τ - Singlet corresponding to three protons, assigned to the methyl aglycon.

This spectrum is more easily understood if the sugar derivative has the C1 rather than the 1C conformation, (see figure 15).

Figure 15.



as follows:

(i) The anomeric proton would seem to be axial rather than equitorial because it appears at about 5.35τ rather than 4.8τ (90); if the doublet may be assigned to this proton then it is strongly coupled with H-2 - as required for a diaxial system.

(ii) Of the protons H-2, H-3, H-4, two appear at lower field than the third and are weakly coupled - results which are consistent with the presence of equatorial protons at positions 3 and 4. The proton at higher field would then be the axial H-2 with the strong coupling with H-1, presumably masked by partial merging with the methyl signal.

DISCUSSION.

The isolation of oxalic acid after ozonolysis, de-esterification and hydrolysis of 2-hydroxyethyl alginate indicates that 4,5-unsaturated products are obtained from the base-catalysed degradation. This is supported by other evidence e.g. ultra-violet spectra and a positive thiobarbituric acid reaction. The isolation of a series of oligosaccharides after column chromatography giving ultra-violet and infra-red spectra characteristic of α,β-unsaturated esters confirms these findings.

The major problems are to decide the identities of the monomer(s) formed in the reaction and of their degradation products. As stated in the introduction to this section the monomer obtained from an enzymic degradation of alginic acid was an α-keto acid. This is very unstable (91) and it is therefore not surprising that it was not detected in the degradation products. A product was isolated (experiment 18) which may have been a degradation product of the α-keto acid. Unfortunately the identity of this substance was not confirmed and it was not obtained again when the separation was repeated, but conditions were more strictly anhydrous the second time.

The formation of products with ultra-violet absorption maxima at higher wavelengths indicates that further elimination is taking place. This has also been reported in the buffer degradation of pectin (69). The product with the ultra-violet absorption maximum at 279mμ could well be 2,3-unsaturated as well as 4,5-unsaturated. The position of the absorption maximum would fit in with this, since the increase in conjugation would be expected to cause a shift to approximately this wavelength (92, p. 143). The fact that in acetate buffer the 279mμ absorption is not observed, but instead an absorption at 235mμ is, can

also be explained by this hypothesis. The acid pH would favour the formation of a keto tautomer (figure 16).

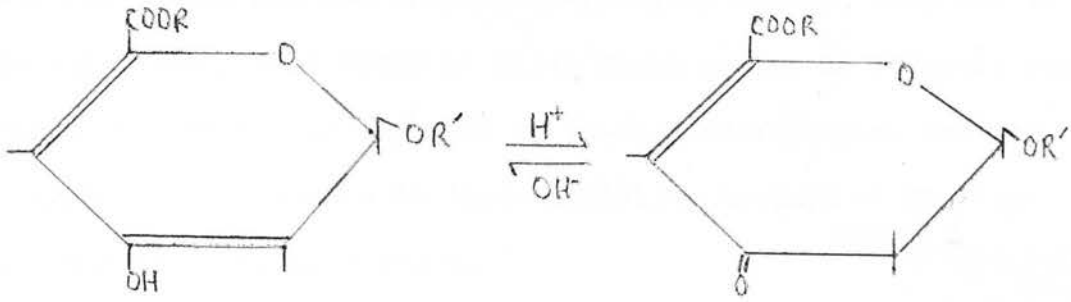


Figure 16.

A keto group does not increase the λ_{max} in α,β-unsaturated ketones (92, p.143) and presumably an analogous situation exists in the case of α,β-unsaturated esters. No reference to this could be found in the literature although such may exist. The second elimination, resulting in the formation of a molecule of water, could also explain why some de-esterification took place in this reaction.

The appearance of ultra-violet absorptions at wavelengths other than those already mentioned i.e. at 240mμ and 262mμ, is not understood.

Differences in results between the two attempts to separate and characterise the products of elimination may have arisen due to the more anhydrous conditions the second time.

Early studies suggested that the elimination reaction was not stereospecific, since elimination, as judged by ultra-violet spectra, was found to have taken place in esters of polysaccharides containing both galacturonic acid and glucuronic acid as the sole uronic acid residues in the molecule. The eliminated groups in these molecules have and have not respectively, a trans anti-parallel arrangement. However, this will require further investigation as the extent of the elimination in these molecules is not known. A comparison of the rate of elimination in guluronic acid-rich and mannuronic acid-rich

alginates was also carried out. Results indicated that the mannuronic acid-rich sample was degraded quicker. Since the eliminated groups in mannuronic ester are not trans anti-parallel whereas they are in guluronic ester, this would at first sight appear to indicate that the trans anti-parallel arrangement is in fact disfavoured. However, results are probably complicated by inaccessibility factors of the type mentioned in Sections A and B.

Elimination studies on model compounds have been started to illuminate the pathway of degradation because it should be easier to characterise the products in these simpler systems. A methyl glycoside of guluronic acid has been prepared (experiment 24(D)) and glycosides of mannuronic acid, glucuronic acid and galacturonic acid are being prepared by other workers for comparative studies. In the meantime the p.m.r. spectrum of methyl β-D-gulopyranosiduronic acid indicates that the sugar probably exists in the 1C conformation in the L-series (see experiment 24(d)). This is important for the overall shape of the polymer chain since the conformation of the monosaccharide units must be known before any attempt can be made in elucidating the polymer conformation by X-ray diffraction or any other means. Unfortunately the configuration of the L-guluronosyl residue in the polymer is unknown. If it has a β-configuration then it will presumably have the same conformation i.e. 1C, as our model compound. However, this need not necessarily be so if the configuration is α. If the 1C conformation did exist in polyguluronic acid then this might explain the shorter fibre repeat distance compared to cellulose (which has the same carbon skeleton, ignoring C-6), in which the glucose units are in the C1 conformation.

SECTION. D.

X-RAY DIFFRACTION STUDIES.

INTRODUCTION.

Chemical methods are essential for elucidating the chemical (primary) structure of polysaccharides, but physical methods have to be employed to obtain information about the secondary and tertiary structures.

For instance, methods involving light-scattering, ultra-centrifugation and viscosity can often give the general shape of polymers in solution (93,94). Such methods are difficult to use for polyelectrolytes (94), and the information obtained is not of a very detailed nature; it also appears likely that polysaccharides will adopt largely random conformations in aqueous solution (94). In the solid state there is a tendency for the polysaccharide chains to exist in conformations which are fixed, unique and regular (95), and therefore more amenable to study. The most powerful of all physical methods for elucidation of molecular shape and structure is X-ray diffraction and this is applicable to the solid state. It has become important to determine these conformations in order to understand the physical and biological properties (96) and chemical reactions of polysaccharides. Problems have arisen in the present study (see Sections B and C) and also in the work of others e.g. limited hydrolysis (13) and limited periodate oxidation (6) of alginic acid, indicating that the rate and extent of heterogeneous reactions carried out on alginic acid appear to be limited. These limited reactions are presumably owing to selectivity of the reagents for certain parts of the solid structure. If this selectivity were understood, then perhaps it could be turned to advantage in industrial and structural studies. As a first step in this

direction it was decided to investigate the chain conformation in the solid state of alginic acid and related polysaccharides by X-ray diffraction.

Previous X-ray diffraction work has in fact been carried out on alginic acid by Astbury (23). This work was confused because it was not known that guluronic acid was a constituent of alginic acid and in fact Frei and Preston (25) have since shown that the work carried out by Astbury was on a guluronic acid-rich sample. They also showed that guluronic acid-rich alginic acid gives a sharper diagram than mannuronic acid-rich alginic acid and that the free acid gives a sharper diagram than the calcium salt.

Previous X-ray diffraction work has been carried out in this laboratory on salts of a sample of K-carrageenan (26) and although the diagrams obtained were good and a certain amount of information was found out about the secondary structure of this sample, the diagrams were not good enough to give a complete picture of the polysaccharide. Accordingly, since some other kappa samples were available it was decided, as an introduction to the work on alginic acid, to look at the potassium salts of these samples and also to look at the potassium salts of the samples after alkali elimination. (Alkali elimination converts any 6-sulphate into the 3,6-anhydro sugar). It was fully expected that the effect of deviation from a perfectly regular primary structure might also be studied with these samples since their primary structures were comprehensively known and appeared suitable for this. This was of great relevance to the work on alginic acid since it is thought that it is the regions of non-regularity which are reactive in the heterogeneous reactions of alginic acid mentioned previously.

Although X-ray diffraction is not a new technique in the study of polysaccharides, no polysaccharide structure has been completely solved by this method. Cellulose was first examined in this way as long ago as 1913 (97), but to this day, the exact nature of the cellulose crystallite molecules is not known. For example, it is not known whether adjacent molecules are parallel or anti-parallel (95) - an uncertainty which has greatly hampered the growth of biogenetic theory.

To obtain the maximum information from polymers, the material must exist in a highly ordered state, either as crystals or as oriented fibres or films. It is significant that most of the polysaccharides which give good X-ray diffraction photographs are the ones which exist naturally in a crystalline state. The prime examples are cellulose, chitin and starch. When polysaccharides are not naturally crystalline they have to be made so and attempts to obtain oriented fibres have met with varying success.

When X-ray fibre diagrams have been obtained there is the problem of their interpretation. In general, fibres show only orientation along the fibre axis, the other two molecular directions being random. The fibre diagram thus resembles the rotation photograph of a single crystal although it usually contains less information. The methods for obtaining unit cell dimensions and symmetry type for a single crystal and a fibre are similar, but one is restricted to a single diffraction photograph for fibres, whereas a crystal can be photographed in its three orientations to obtain information.

The difference between the two cases when making a more refined interpretation is profound. For single crystals there are two courses: (a)-from the intensities of the spots, the distribution of electron density in the unit cell is calculated and matched with a model of the

compound. The calculation is not straightforward however, since the phases of the diffracted X-rays are not known; (b)-from a model of the compound, unit cell co-ordinates are assigned to each atom, then the intensities are computed and matched with the observed intensities. For fibres, there is generally an insufficient number of reflections to calculate the electron density distribution (95), and so one is forced to the second, more empirical, alternative. That is, to postulate a likely conformation of the polymer, and from the proposed atomic positions, compute the spot intensities; and if the proposed structure is correct, then agreement between the observed and calculated intensities is good. Thus one has to know the primary structure of the polysaccharide before making a detailed interpretation of the X-ray fibre diagram.

EXPERIMENTAL.

Experiment 25. Alkaline modification of K-carrageenan.

It has been shown that K-carrageenan contains an alternating structure of α-1,3 and β-1,4-linked units (98,99). The 3-linked units are exclusively galactose 4-sulphate, while the 4-linked units are predominantly derivatives of 3,6-anhydro galactose, with a small proportion of galactose 6-sulphate derivatives. The presence of the 6-sulphate residues therefore introduces an irregularity into the basic alternating structure. The 3,6-anhydro derivatives will be present in the 1C conformation whereas the galactose 6-sulphate residues will be present in the C1 conformation so that the latter will undoubtedly cause a kink in the polysaccharide chain. This irregularity may be removed by the formation of 3,6-anhydro galactose derivatives from their corresponding 6-sulphate derivatives by alkali modification (98,99). It was hoped that this would improve the X-ray fibre diagrams of samples of K-carrageenan.

Various K-carrageenan samples have been shown to contain different quantities of sulphate ester at the C-2 positions of the 4-linked unit (100) and as yet the effect of this substituent on the secondary structure is not known. It was hoped to gain insight into this in the present work. Four samples of K-carrageenan were available, their structures were accurately known and they appeared suitable for investigating the above problems.

The K-carrageenan samples and the relevant data about them are listed in Table 7.

Table 7.

Sample	% 3,6-anhydride that is 2-sulphated	% 4-linked units that are:- (i) 6-sulphated only	(ii) 2,6-disulphated
TD-Kappa	20	4.2	1
REX 5104	5	1	0
Eucheuma cottonii	0	7.2	0
E. spinosum	100	0	9.5

Further details about these samples are given in N. S. Anderson's Thesis (26).

The alkali modification of these samples was carried out as follows:-

The potassium salt of K-carrageenan (100mg) was dissolved in water (25ml) with sodium borohydride (50mg), and the solution was left overnight at room temperature. The solution was then made 1N to sodium hydroxide and a further 50mg of sodium borohydride was added. The solution was heated at 80° for 5 hours. After cooling, the solution was dialysed. The required salt of the alkali modified sample was then obtained by passing the above solution through IR-120 in its appropriate salt form. A 15cm column of diameter 1.5cm was used and the exchange resin was prepared by passing a solution of the appropriate chloride through the column until the effluent solution was neutral. The column was then washed thoroughly before use. The following salt forms were prepared:- lithium, ammonium, sodium, potassium, rubidium, caesium and calcium.

Experiment 26. The preparation of oriented fibres for X-ray diffraction.

Fibres of soluble polysaccharides were prepared by essentially the same method as described by Dr. N. S. Anderson (26). In detail, two glass rods were mounted in plasticene with one end held rigid and the

other on a moveable support, such that the rods could be gently pulled apart. A drop of a warm viscous solution of the polysaccharide was then placed over the ends of the rods (Stage A, figure 17) and before the solution gelled one rod was gently moved to a final position (about 0.3cm back from the fixed rod) to give a symmetrical elongated drop (Stage B). The solution gelled on cooling and was then left to dry out to a fibre (Stage C).

Figure 17.

support rods

droplet

Stage A Stage B Stage C

The conditions of drying were controlled by mounting the glass rods in a sealed cell and forming the fibres at known temperature and humidity. A saturated solution of sodium bromide, giving a relative humidity of 57%, was placed in the cell and the cell was left at 1° overnight. On removing the glass rods from the plasticene and pulling along the fibre axis, the fibre was usually detached from one of the glass rods to give an oriented fibre attached to a single glass support. The fibre was then ready for X-ray diffraction.

In the case of polysaccharides which were insoluble e.g. alginic acid, a fibre of a suitable soluble salt was prepared. The two glass rods supporting the fibre were then fixed onto another glass support (figure 18) using Araldite adhesive.

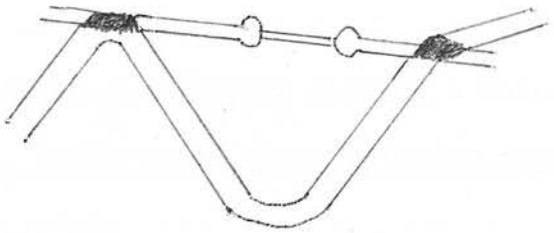


Figure 18.

After the Araldite was dry the main glass support was attached to a glass rod, using plasticene, so that it could be immersed in the appropriate solution. For example, in the preparation of fibres of alginic acid the soluble sodium salt fibre was prepared and was then immersed in 1N hydrochloric acid overnight. After washing overnight in standing water, the fibre was left to dry for 2 hours at room temperature and was then ready for X-ray diffraction as before.

All the samples examined of salts of K-carrageenan and the corresponding alkali modified samples gave stiff gels and strong fibres, except for the calcium salt of alkali modified E. spinosum which gave rather brittle fibres, although it gave an extremely stiff gel.

The soluble salts of alginic acid in general gave viscous gels from which strong fibres were prepared. Fibres of alginic acid from a mannuronic acid-rich sample, a guluronic acid-rich sample and a sample with an intermediate uronic acid composition were prepared and photographed. It was found that the sample which gave the clearest diagrams was the guluronic acid-rich sample. This is consistent with the work of Frei and Preston (25) and accordingly this sample of alginate (from L. cloustonii) was used to prepare the various salt form fibres for X-ray diffraction. The calcium salt was not prepared since this has previously been shown (25) to give a poor X-ray fibre diagram.

An attempt to form fibres from the soluble sodium salt of sun-

flower pectic acid (containing 99.2% galacturonic acid) was unsuccessful although a strong gel was obtained.

Experiment 27. Preparation of an oriented aqueous gel for X-ray diffraction studies.

It was desirable to find out if aqueous gels of polysaccharides in general and the potassium salt of alkali modified carrageenan from E. spinosum in particular, contain regions of order which are possibly responsible for the gelling properties of the system. To investigate this, a modification of the method used for preparing oriented fibres (see experiment 26) was used, whereby the drying out of the gel was stopped before a fibre was obtained and the gelled state was maintained while the X-ray diffraction photograph was being taken. The following procedure was devised:-

Two glass rods were vertically aligned with each other using plasticene to keep them in position. The upper rod had a larger bulb at its extremity than the lower rod. A fine glass capillary was attached to the upper rod, again using plasticene, so that it could slide up and down the glass rod (figure 19).

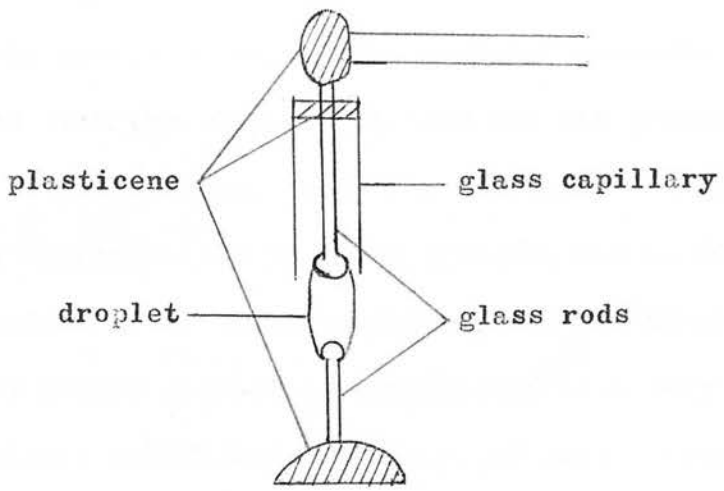


Figure 19.

A drop of a viscous solution of the polysaccharide (3%, w/v) was

then placed over the ends of the rods. On cooling, a gel was formed and on standing at room temperature for approximately 1 hour the gel was sufficiently thick for X-ray diffraction studies. The glass capillary was then gently slid down the upper rod and plugged into the plasticene in which the bottom rod was standing. A piece of damp cotton wool was also introduced into the glass capillary before plugging with the plasticene. On removing the upper rod from its plasticene support the gel was ready for X-ray diffraction. The appearance and plastic character of the gel after diffraction confirmed that it had not dried out to a fibre while the photograph was being taken.

Experiment 28. The X-ray fibre diagrams obtained.

The fibre diagrams were obtained with a Charles Supper Precession Camera modified for fibre diffraction work by fitting a fine collimator made from lead glass capillary. The film was contained in a flat-plate camera. No precautions against air scatter were taken. To obtain the X-ray fibre diagram, the fibre was tilted by 10° out of the normal to the X-ray beam. The fibre to the film distance was 6cm.

The diagrams of the potassium salts of the K-carrageenan samples and their corresponding alkali modified products were obtained and compared with the diagram obtained for the potassium salt of REX-5104 by Dr. N. S. Anderson. Those for REX-5104, TD-Kappa and E. cottonii were of approximately the same standard and no detectable improvement was obtained after alkali modification. The diagram for E. spinosum was very poorly defined in comparison, with only traces of sharp spots; after alkali modification however, it gave a remarkably clear and well-defined diagram (figure 20). Accordingly it was decided to study further the alkali modified sample of E. spinosum - in future this

carrageenan from E. spinosum will be referred to as iota carrageenan.

The lithium, ammonium, sodium, rubidium, caesium and calcium salts of the alkali modified iota sample were made and fibres were prepared and photographed. The lithium, ammonium and sodium salts again gave very clear diagrams (figure 20). The exposure time for these photographs was approximately 5 hours. In the case of the rubidium salt only a weak, rather indistinct photograph was obtained even after a 12 hour exposure period. After this time background scatter was pronounced, presumably by the heavy rubidium ions in the more disordered parts of the structure. To diminish the effect of absorption by the rubidium ions thinner fibres were prepared and photographed, but little if any improvement was obtained. The photographs from the caesium salt were extremely poor. The calcium salt although giving a very firm gel gave rather poor fibres and a poor fibre diagram.

To obtain better definition of the reflections on the fourth and fifth layer lines of the fibre diagram of the potassium salt of the alkali modified iota sample, the angle of tilt was adjusted to 13.6° and 17.1° (calculated by Dr. M. Harding) respectively. This showed that there were no meridional reflections on these layer lines.

The diagram of the oriented gel of the potassium salt of alkali modified iota carrageenan had a strong background due to scatter of the X-ray beam by the glass capillary and water. However, spots were clearly visible which corresponded to the innermost spots on the zero and first layer lines of the fibre diagram. The layer line spacing was similar to before, but the spots were approximately 15% closer to the meridion. The meridional spot on the third layer line was also visible and also a spot on the second layer line.

The lithium, ammonium, sodium and potassium salt fibres of alginic

acid gave X-ray fibre diagrams very similar to each other. As with alkali modified iota carrageenan, the rubidium salt fibre gave a relatively diffuse diagram and the caesium salt fibre diagram was very diffuse.

Experiment 29. Measurement of fibre densities.

Densities were measured by flotation in mixtures of carbon tetrachloride and methyl iodide or carbon tetrachloride and benzene. The following densities were obtained for salts of alkali modified iota carrageenan.

Ammonium salt 1.57g/ml

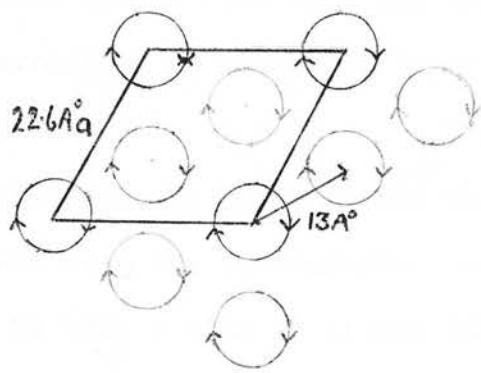
Potassium salt 1.72g/ml

DISCUSSION.

The well-defined fibre diffraction photographs of salts of alkali modified iota carrageenan have been interpreted by Dr. M. Harding. The results are summarised below and are compared to those obtained with a typical K-carrageenan sample, REX-5104.

The different iota salts have the same fibre axis repeat distance of 13.0Å, within a few percent. They all show a reflection on the meridion in the third and sixth layer lines but not on other layers. This would correspond to a helical arrangement with three disaccharide units per turn - effectively a three-fold screw axis. The different salts give diagrams which are clearly related, indicating that the different salts are isostructural, yet there are definite intensity changes when the cations are exchanged. These can be used to find the cation contributions to the pattern and work in this direction is proceeding. The maxima of the iota fibre diagrams were sharp enough to suggest regular lateral packing of the helices in a hexagonal cell of side 22.6Å. A cell of the type shown in figure 21 would account for almost all the observed diffraction pattern, although beyond the first three or four orders it is not possible to index reflections unambiguously.

Figure 21.



The projection is down the helix axis. The helices are arranged in hexagonal packing, but they are not packed in register - each row of helices is displaced up the C axis by one-third of the unit cell relative to adjacent rows.

The number of disaccharide units per fibre repeat has been deduced from the cell dimensions and measured density (the molecular weight of the disaccharide plus an assumed water content of 15% (101) was used in these calculations) and assuming the packing shown in figure 21. Values of 3.08 and 3.15 were found for the ammonium and potassium salts respectively which were close to three disaccharide units per 13Å fibre repeat. These deductions are independent of those made for the intensity distributions on the layer lines, but are consistent with them.

If we now consider K-carrageenan salts we find that they are not so well oriented, but they all have a fibre repeat distance of 24.6Å and meridional reflections on the third, sixth, ninth and twelfth layer lines - again characteristic of a three-fold screw axis.

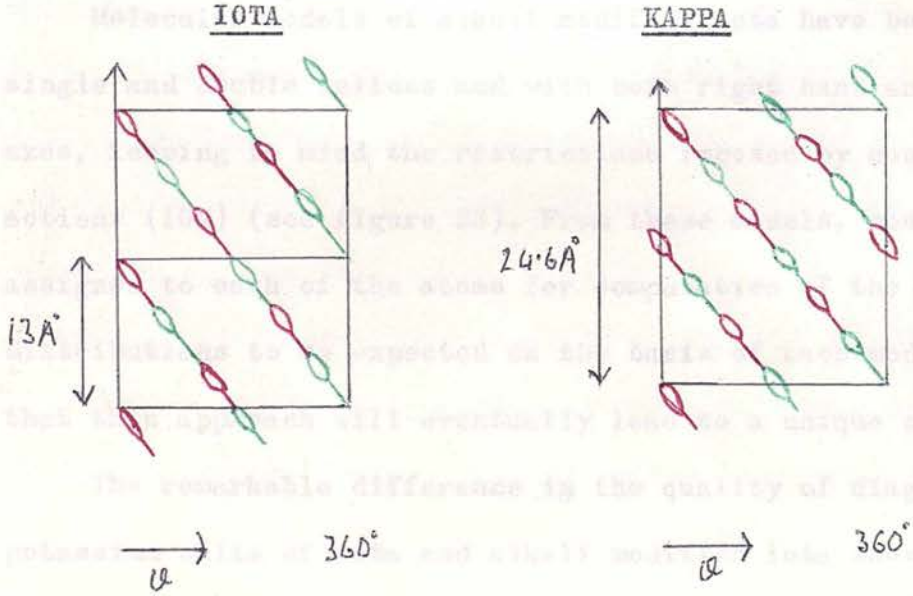
The intensity distributions (obtained from densitometer traces by Dr. M. Harding and Mr. J. W. Campbell) within the layer lines for K-carrageenan and alkali modified iota carrageenan show clear relationships between the two series. The C axis of the kappa fibres is about twice that of the iota ones and therefore we expect each iota layer line to correspond to alternate layer lines of kappa. This in fact has been observed.

The orientation of the kappa fibres was not good enough for a unit cell to be derived from the photographs. However a cell of the type shown in figure 21 with a side of around 20Å is entirely consistent with the observed patterns. There is also very good agreement with

the volume per residue allowed by this cell and the measured densities. A value of 6.6 disaccharide units per fibre repeat was obtained for kappa - again the result is independent of those made from the intensity distributions on the layer lines, but is consistent with them.

Schematic drawings are shown in figure 22 of the types of arrangement which will be tested further by Fourier transforms.

Figure 22.



The two types of unit are represented by different coloured blobs: each drawing represents a projection of a double helix onto a cylinder surface which is then opened out. (To form the double helix, the drawing is folded into a cylinder). In the iota, each chain repeats in 26Å, but the second chain is staggered with it so that the crystallographic repeat is half of this chain repeat - 13Å. An actual model of this double helix can be built very comfortably and is found to have an average external diameter of about 12Å. It would therefore fit nicely into our unit cell in which the helices are separated by

13Å.

The kappa model is similar but with a slightly shorter repeat distance. The relation between the chains is not known: however, they are not exactly staggered and the crystallographic repeat is therefore the same as the chain repeat. It would seem that the removal of one sulphate group from each disaccharide unit, to convert iota into kappa, alters the minimum energy position of each chain with respect to its partner in the double helix.

Molecular models of alkali modified iota have been built of both single and double helices and with both right hand and left hand screw axes, keeping in mind the restrictions imposed by non-bonded interactions (102) (see figure 23). From these models, coordinates can be assigned to each of the atoms for computation of the intensity distributions to be expected on the basis of each model. It is hoped that this approach will eventually lead to a unique conformation.

The remarkable difference in the quality of diagrams between the potassium salts of iota and alkali modified iota shows the marked effect of a slight disruption (9.5% of the 4-linked units are present as 6-sulphate in the non-alkali modified sample) in the strictly alternating structure. In contrast, the diagrams of kappa showed little improvement after alkali modification. In the case of K-carrageenan there is fractionation evidence (26) that the 6-sulphate may be rather randomly distributed so that there may be sufficient non-kinked parts of chain to show some ordered structure. The absence of this pattern in iota suggests a more uniform distribution of 6-sulphate. A model was also built with a single 3,6-anhydro derivative replaced by a 6-sulphate derivative. A regular helix containing this unit could not be constructed but a kinked helix could (figure 23). This kink

explains the poor diagrams obtained from the non-alkali modified sample.

The extremely diffuse diagram obtained from the calcium salt of alkali modified iota suggests that the order produced in the solid state by monovalent cations is not produced by divalent cations.

The diagram obtained from the oriented gel indicates that in this aqueous system the chain conformations and to some extent the packing of chains, is similar to the dried fibres. The repeat distance along the molecular axis is similar to that for the corresponding fibre. However, the lateral distance between double helices is increased, no doubt by increasing the helix diameter by hydration. In the oriented gel the helices have been aligned but in a normal gel they are presumably present but randomly oriented. This suggests that the formation of localised double helices by a single polymer with several other polymer chains, when repeated throughout the structure, forms the rigid support for interstitial water molecules so that the whole is held together. When the gel is heated there will be a decrease in order in the system i.e. the double helices will be disrupted, giving random coils, so that the gel will lose its points of support and will therefore collapse.

The fibre diagrams of soluble salts of alginic acid were much less detailed than that of alginic acid itself and since a diagram of guluronic acid-rich alginic acid has been comprehensively examined (23) it was decided not to pursue studies any further.

The sodium pectate sample would not give a fibre and it is presumed that the cohesive forces between molecules in the solid state were not sufficient to hold the structure together.

The alginate esters which have been referred to in sections B and C all gave fibre diffraction diagrams (defined arcs). They might there-

fore contain regions of crystallinity and this might account for the inaccessibility to reagents of certain parts of these molecules, as described in these sections.

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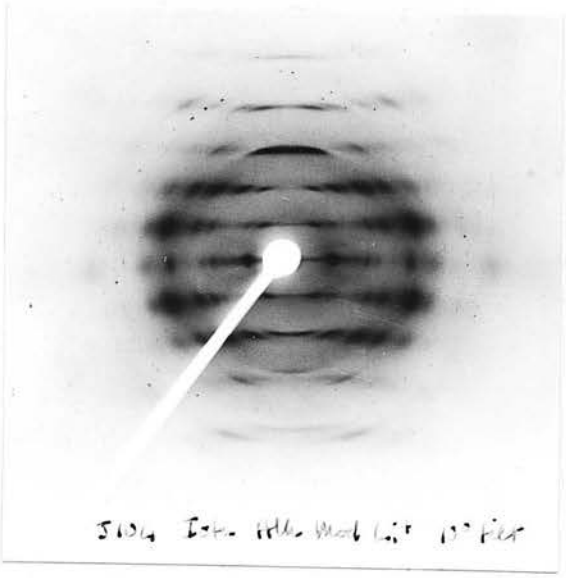
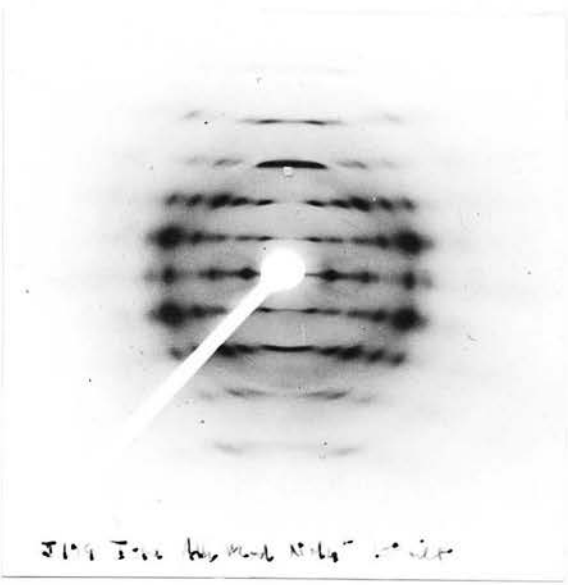
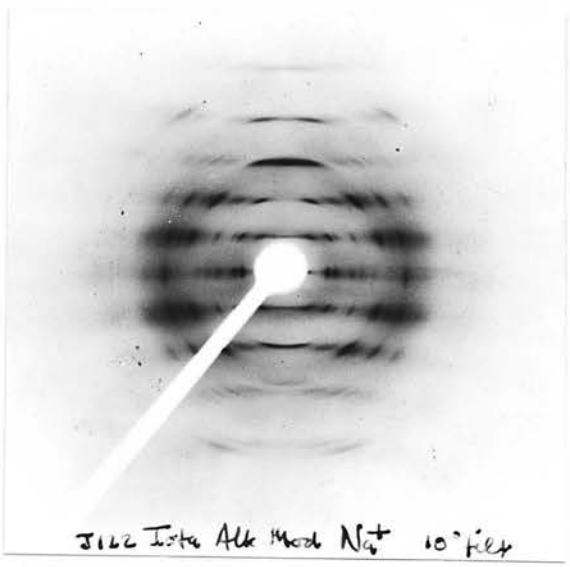


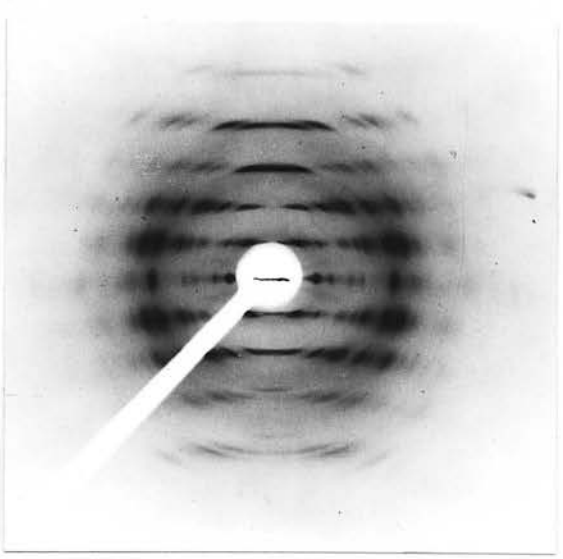
LITHIUM SALT



AMMONIUM SALT



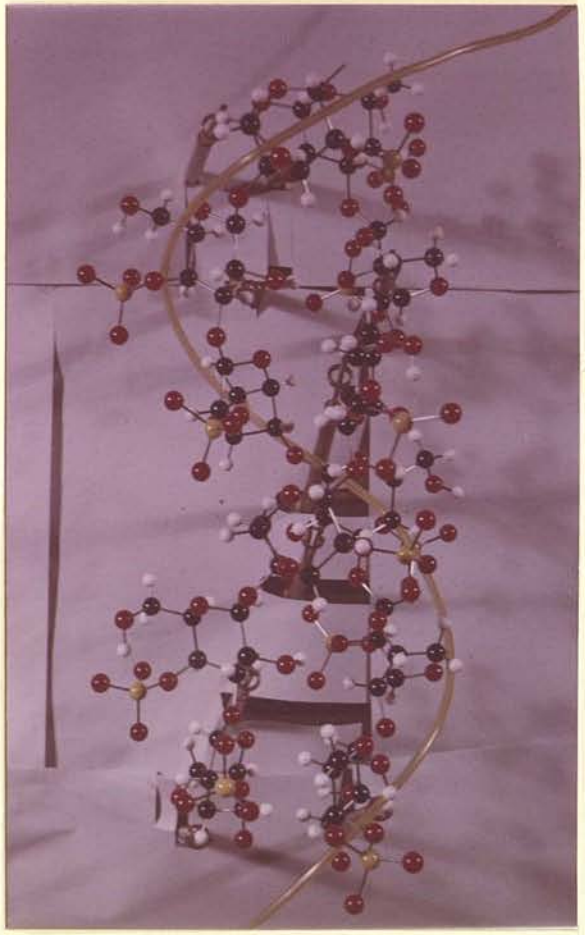
SODIUM SALT

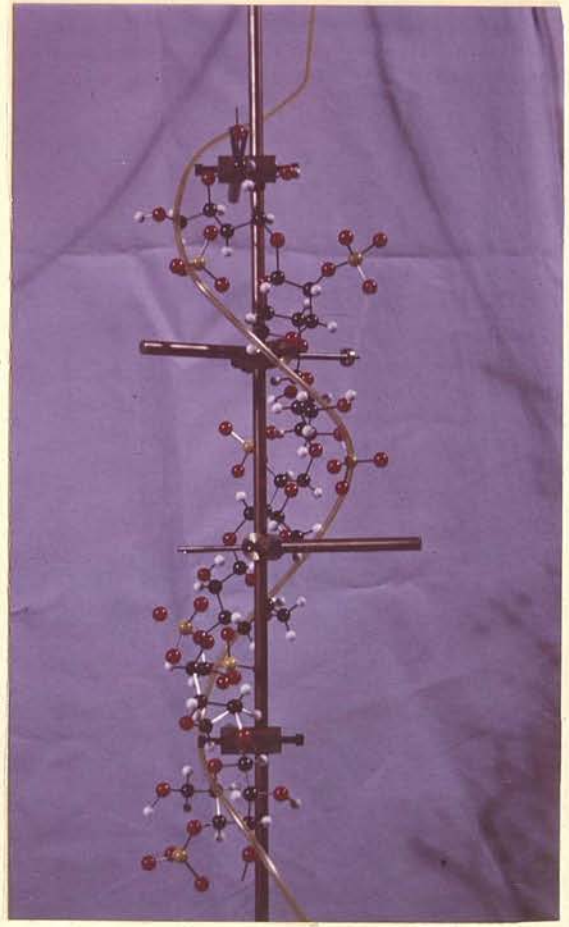


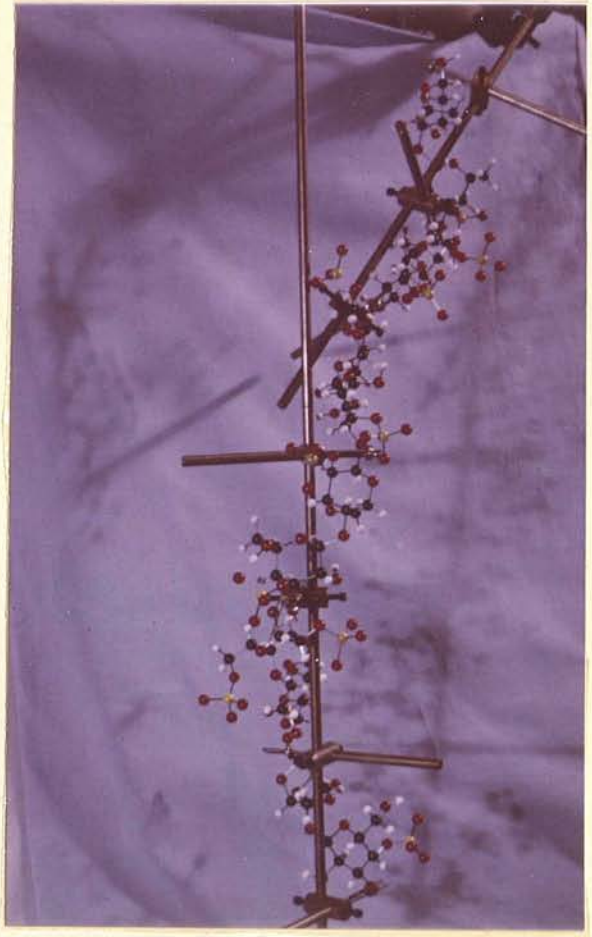
POTASSIUM SALT

X RAY FIBRE DIAGRAMS OF SALTS OF ALKALI MODIFIED IOTA CARRAGEENAN

Figure 20.







RIGHT-HANDED DOUBLE HELIX LEFT-HANDED SINGLE HELIX RIGHT-HANDED SINGLE HELIX CONTAINING A KINK

Black balls represent carbon atoms, red represent oxygen, white represent hydrogen, yellow represent sulphur. The projected angle between adjacent horizontal supports is 120°.

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Carboxyl-reduction of Alginic Acid and other Polysaccharides

By D. A. Rees and J. W. B. Samuel

Chemistry Department, The University, West Mains Road, Edinburgh, 9

Although the reduction of most carboxylic acids and esters to the corresponding primary alcohols can be carried out conveniently and quantitatively by the use of suitable hydrides, such reactions are not easy for polysaccharides.[1] These substances are insoluble in the solvents normally used and although their methyl ethers are often soluble and can be reduced, the products cannot be recovered in the unsubstituted form. Aqueous media can be used when the ester is reduced with sodium or potassium borohydride, but ester hydrolysis also occurs and it is therefore necessary to repeat the esterification and reduction several times. Heterogeneous reduction[2] is usually incomplete because not all the molecules in the solid phase are accessible to the reagent. Another method[2] that has been successful in some instances, is to make the polysaccharide soluble in the reaction solvent by acetylation or propionylation and then to reduce the carboxylic acid groups with diborane; the solubilising groups can be removed afterwards. We wished to reduce alginic acid as the first step in further structural studies,[3] but unfortunately the diborane reduction of alginic acid is incomplete,[2,4] and a highly undesirable side reaction occurs under the best conditions for rapid reduction.[4] We report now two methods by which alginic acid, and probably also other polysaccharides, may be conveniently and quickly reduced.

The first is based on the observation[5] that trimethylsilyl ethers react very slowly with lithium aluminium hydride. Alginic acid was converted into the methyl ester with diazomethane, trimethylsilylated[6] and then treated with lithium aluminium hydride in cold tetrahydrofuran. The trimethylsilyl groups confer solubility on the polysaccharide derivative, and are readily removed with water after the reaction. The product separates slowly as reduction proceeds. This method is inferior to that described below because a significant degree of methyl etherification occurs during the treatment with diazomethane.[7] It may, however, be the method of choice if a polysaccharide methyl ester is available by another route. Other easily prepared esters of alginic acid (2-hydroxyethyl and 2-hydroxypropyl esters) cannot be so reduced because they do not give soluble trimethylsilyl derivatives by the methods we have tried.

We have also found that reduction can be carried out heterogeneously, provided that precautions are taken to ensure that all the polysaccharide is completely accessible to the reducing agent. This is conveniently achieved using the acetate of the carboxyl-esterified polysaccharide. Presumably the reductive deacetylation that occurs simultaneously with the reduction of the uronate units leaves the polysaccharide phase with an open structure that is completely penetrable by the reagent. 2-Acetoxyethyl di-*O*-acetyl-alginate and methyl di-*O*-acetylalginate have been used successfully in this reaction. The latter derivative was prepared by the action of diazomethane on di-*O*-acetylalginic acid.[8] Lithium aluminium hydride cannot be used as the reducing agent, presumably because the derivatives it forms near the surface of the polysaccharide phase protect molecules in the interior from reduction. Lithium borohydride in used in boiling tetrahydrofuran for 16 hours. The reduced polysaccharide is readily isolated by dialysis and freeze-drying. In a typical experiment, methyl di-*O*-acetylalginate (0·375 g.) gave a product (0·168 g. 76%) which had a uronic anhydride (decarboxylation) content of 4·7%, compared with the value of 3·5% that is reported for mannose.[9] There was no carbonyl absorption in the infrared spectrum and barely detectable traces of uronic acids were shown by paper chromatography and paper electrophoresis after hydrolysis. In order to achieve complete reduction by this method it is necessary to achieve complete carboxyl-esterification in the previous stage. The complete 2-hydroxyethyl-esterification of alginic acid requires very prolonged treatment with ethylene oxide in salt free solution, and reduction via the methyl ester might therefore be more convenient.

Preliminary experiments with the pectic polysaccharides from white mustard[10] suggest that the heterogeneous reduction method might be widely applicable in polysaccharide chemistry.

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